THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of: Brenneisen, et al.	Confirmation No.: 3801
Application No.: 10/580,186	Group Art Unit: 1654
Filing Date: September 21, 2007	Examiner: Phyllis G. Spivack
For: PLANT EXTRACTS FOR THE TREATMENT OF INCREASED BONE RESORPTION	Attorney Docket No.: 8588-US

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANT'S APPEAL BRIEF

Sir:

Appellant submits this Appeal Brief in support of the Notice of Appeal filed February 23, 2011. This Appeal is taken from the Final Rejection dated November 24, 2010 and the Advisory Action dated February 4, 2011.

I. REAL PARTY IN INTEREST

The real party in interest for the above-identified patent application on Appeal is Universitat Bern by virtue of an Assignment dated September 21, 2007 and recorded at reel 019860, frame 0361 in the United States Patent and Trademark Office.

II. RELATED APPEALS AND INTERFERENCES

Appellant's legal representative and the Assignee of the above-identified patent application do not know of any prior or pending appeals, interferences or judicial proceedings which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

III. STATUS OF CLAIMS

Claims 1-10, 12-24, 26-33 and 36-47 are pending in the above-identified patent application. Claims 1-9, 29-33, 36-37 and 41-44 were previously withdrawn from consideration. Claims 11, 25, 34 and 35 were previously canceled without prejudice or disclaimer. Claims 10, 12-24, 26-28, 38-40 and 45-47 stand rejected. Therefore, Claims 10, 12-24, 26-28, 38-40 and 45-47 are being appealed in this Brief. A copy of the appealed claims is included in the Claims Appendix.

IV. STATUS OF AMENDMENTS

The Examiner mailed a non-final Office Action on July 16, 2010, in which the Examiner rejected Claims 10, 12-24, 26-28, 38-40 and 45-47 under 35 U.S.C. §102(b). Appellant responded to the non-final Office Action in which Appellant addressed the anticipation rejection. The Examiner mailed a final Office Action on November 24, 2010, in which the Examiner maintained the rejection. On January 20, 2011, Appellant filed a Response to the final Office Action in which Appellant addressed the anticipation rejection. The Examiner mailed an Advisory Action on February 4, 2011. Appellant filed a Notice of Appeal on February 23, 2011. A copy of the non-final Office Action, final Office Action and Advisory Action are attached hereto as Exhibits A, B and C, respectively.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the invention by way of reference to the drawings and specification for each of the independent claims and each means plus function claim may be found in Appendix B to this Brief.

Independent Claim 10 is directed to a nutritional composition comprising a γ-glutamyl-peptide (page 3, line 31-page 4, line 12) selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide (page 3, lines 5-9; page 3, line 31-page 4, line 12), and combinations thereof, a nutritionally acceptable carrier (page 4, lines 5-12; page 18, lines 4-19), and a fat source (page 13, lines 13-17 and 28-32).

Independent Claim 24 is directed to a pharmaceutical composition in single unit dose form (page 17, lines 15-16), comprising a γ-glutamyl-peptide (page 3, line 31-page 4, line 12) selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide (page 3, lines 5-9; page 3, line 31-page 4, line 12), and combinations thereof, a pharmaceutically acceptable carrier (page 17, line 19-page 19, line 2), and a fat source (page 13, lines 13-17 and 28-32).

Although specification citations are given in accordance with C.F.R. 1.192(c), these reference numerals and citations are merely examples of where support may be found in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from the specification into the claims. Pointing out specification support for the claim terminology as is done here to comply with rule 1.192(c) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the references numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 10, 12-24, 26-28, 38-40 and 45-47 are rejected under 35 U.S.C. §102(b) as being anticipated by WO 98/50054 to Mühlbauer ("*Mühlbauer*") as being evidenced by Kuttan et al. ("*Kuttan*") and as evidence by J. Agric. Food Chem., 2005, 53(9): 3408-3014 to Wetli et al. ("*Wetli*"). Copies of *Mühlbauer*, *Kuttan* and *Wetli* are attached hereto as Exhibits D, E and F in the Evidence Appendix.

VII. ARGUMENT

A. LEGAL STANDARDS

Anticipation under 35 U.S.C. §102

Anticipation is a factual determination that "requires the presence in a single prior art disclosure of each and every element of a claimed invention." *Lewmar Marine, Inc. v. Barient, Inc.*, 827 F.2d 744, 747 (Fed. Cir. 1987). Moreover, "[a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a *single* prior art reference." *Verdegaal Bros. v. Union Oil of California*, 814 F.2d 628, 631 (Fed. Cir. 1987) (emphasis added).

Federal Circuit decisions have repeatedly emphasized the notion that anticipation cannot be found where less than all elements of a claimed invention are set forth in a reference. See, e.g., *Transclean Corp. v. Bridgewood Services, Inc.*, 290 F.3d 1364, 1370 (Fed. Cir. 2002). In this regard, a reference disclosing "substantially the same thing" is not enough to anticipate. *Jamesbury Corp. v. Litton Indust. Prod., Inc.*, 756 F.2d 1556, 1560 (Fed. Cir. 1985). A reference must clearly disclose each and every limitation of the claimed invention before anticipation may be found.

To establish inherent anticipation, the Federal Circuit has stated that "extrinsic evidence 'must make clear that the missing descriptive matter is *necessarily* present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *Inherency, however, may not be established by probabilities or possibilities.* The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient.'" *In re Robertson*, 169 F.3d 743, 745 (Fed. Cir. 1999) (emphasis added).

In relying on inherency, the Patent Office requires an examiner to supply an applicant with a "basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic *necessarily* flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis added). If the examiner is successful in showing a sound basis, for example, that the products of the applicant and the prior art are the same, the burden then shifts to the applicant to show that they are not. See, MPEP 2112.

B. THE CLAIMED INVENTION

Independent Claim 10 is directed to a nutritional composition comprising a γ-glutamyl-peptide, a nutritionally acceptable carrier, and a fat source. The γ-glutamyl-peptide is selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, or combinations thereof.

Independent Claim 24 is directed to a pharmaceutical composition in single unit dose form, comprising a γ-glutamyl-peptide, a pharmaceutically acceptable carrier, and a fat source. The γ-glutamyl-peptide is selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof.

C. THE REJECTION OF CLAIMS 10, 12-24, 26-28, 38-40 AND 45-47 UNDER 35 U.S.C. §102(b) SHOULD BE REVERSED BECAUSE *MÜHLBAUER* FAILS TO DISCLOSE OR SUGGEST EACH AND EVERY ELEMENT OF THE PRESENT CLAIMS

The Examiner alleges that *Mühlbauer* discloses every element of the present claims, as evidenced by *Kuttan* and *Wetli*. Appellant respectfully submits that the anticipation rejection in view of *Mühlbauer*, as evidenced by *Kuttan* and *Wetli*, is improper and traverses the rejection for at least the reasons set forth below.

Independent Claims 10 and 24 recite, in part, nutritional and pharmaceutical compositions, respectively, comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier, and a fat source. Appellant has surprisingly found that the active constituent of allium responsible for the bone resorption inhibiting effect may be found in a hydrophilic, ethanolic extract of allium such as allium cepa. The active constituent having a potent inhibitory effect on bone resorption was identified as a γ-glutamyl-peptide, for example a γ-glutamyl-alkyl-cysteine sulfoxide or γ-glutamyl-alkenyl-cysteine sulfoxide, or a γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide. See, specification, page 7, lines 32-37. In contrast, *Mühlbauer* fails to disclose every element of the present claims.

For example, *Mühlbauer* fails to disclose or suggest nutritional and pharmaceutical compositions, respectively, comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier, and a fat source as required, in part, by independent Claims 10 and 24. Instead, *Mühlbauer* is entirely directed to plant extracts for the treatment of increased bone resorption. See, *Mühlbauer*, Abstract. The Examiner asserts that *Mühlbauer* teaches a nutritional composition comprising all of the active components of the instant claims. See, final Office Action, page 4, lines 14-16. Appellant respectfully disagrees, however, and submits that the Examiner seems to be ignoring the tenants of patent office practice and legal precedent previously outlined by Appellant in the Response to the non-final Office Action and the Response to the final Office Action.

For example, the Manual of Patent Examining Procedure clearly states that "[a] genus does not always anticipate a claim to a species within the genus. However, when the species is clearly named, the species claim is anticipated no matter how many other species are additionally named." *Ex parte A*, 17 USPQ2d 1716 (Bd. Pat. App. & Inter. 1990). Indeed, the disclosure of a large genus rarely anticipates a narrowly claimed species.

Further, in the Court of Customs and Patent Appeals case of *In re Petering*, a test for determining whether a disclosed genus is sufficiently small enough to anticipate a claimed species was established. 301 F.2d 676, (CCPA 1962). The application at issue in *Petering* contained claims to a particular species of compound. The Examiner cited a reference disclosing a chemical genus, which included the claimed species, having a limited number of substituent groups that represented either hydrogen or alkyl radicals, and an R group containing an OH group. The court held that this formula alone could not anticipate the claimed species because there were too many compounds within this disclosed genus - the genus was too large. The reference, however, also disclosed preferred substituent groups, which included about twenty compounds defining a subgenus. The court found that one of ordinary skill in the art would have been informed enough by the reference to "at once envisage" each member of the subgenus, which included the claimed species. *Id.* Accordingly, the genus-species anticipation test states that a genus anticipates a species if one of ordinary skill in the art is able to "envisage" the claimed species within the disclosed genus. This test was later confirmed by the CCPA in *In re Schauman*, 572 F.2d 312, (CCPA 1978).

Recent Federal Circuit case law has confirmed that the *Petering* and *Schauman* analysis remains the test when considering whether or not a prior art document's disclosure of a genus anticipates a claimed species. See, *Sanofi-Synthelabo v. Apotex, Inc.*, 550 F.3D 1075, 1084 (Fed. Cir. 2008) and *Eli Lilly & Co. v. Zenith Goldline Pharms., Inc.*, 471 F.3d 1369, 1376 (Fed. Cir. 2006) (citing *Petering* and *Schauman* and emphasizing that the disclosure of a broad genus can be narrowed to a specific group of compounds if the reference also discloses preferred embodiments or compounds). As such, Appellant submits that, although *Mühlbauer* discloses the genus *allium* and mentions *allium cepa*, *Mühlbauer* fails to anticipate the present claims because the genus *allium cepa* is too large for the skilled artisan to envisage a γ-glutamyl-peptide extracted from *allium cepa*, let alone a specific γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof as required, in part, by currently amended independent Claims 10 and 24.

Further, *Kuttan* and *Wetli* fail to disclose or suggest nutritional and pharmaceutical compositions, respectively, comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier, and a fat source as required, in part, by independent Claims 10 and 24.

The Examiner asserts that *Kuttan* demonstrates that the "γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide isolated from sandal (Santalum album L.) is the same as the protein isolated from onion (Allium cepa)." See, final Office Action, page 5, lines 12-14. Appellant respectfully disagrees. Instead, Appellant notes that *Kuttan* expressly states that "[c]ircular dichorism measurements established that the sulfoxide group in the sandal and onion peptides are of opposite configurations." See, *Kuttan*, page 4394, column 2. The skilled artisan would immediately appreciate that stereoisomers of the same compound can have widely varying properties including, for example, efficacy in treating or preventing diseases or conditions characterized by bone resorption. Indeed, *Kuttan* also expressly states that "[d]ifferences in peak intensities [of the peptide derived from sandal and from onion] may be due to diastereoisomerism or variations in hydration. The onion peptide is extremely hygroscopic. While the elemental analysis of the sandal peptide fits a monohydrate, it did not appear to be particularly hygroscopic." See, *Kuttan*, page 4396, bottom of column 1 to top of column 2.

Kuttan also states that "[t]he γ-L-glutamyl peptide . . . of S-(1-propenyl)-L-cystein sulfoxide . . . is the principal γ-glutamyl peptide of onion (Allium cepa) . . . being accompanied

by lesser amounts of γ-glutamyl-S-(2-carboxypropyl)cystein . . . and S-methylcysteine . . . among others." See, *Kuttan*, page 4397, 1st paragraph of Discussion. As such, it is clear that the sandal peptide and the onion peptide are not, in fact, the same compound, as alleged by the Examiner. Instead, the skilled artisan would immediately appreciate that the stereoisomers of the sandal and onion peptides most likely have widely varying properties that could include efficacy for the treatment or prevention of diseases or conditions that are characterized by increased bone resorption.

Wetli is cited by the Examiner solely for the molecular mass of gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide. See, Office Action, page 7, line 17-page 8, line 3.

Further, anticipation is a factual determination that "requires the presence in a single prior art disclosure of each and every element of a claimed invention." *Lewmar Marine, Inc. v. Barient, Inc.*, 827 F.2d 744, 747 (Fed. Cir. 1987) (emphasis added). Federal Circuit decisions have repeatedly emphasized the notion that anticipation cannot be found where less than all elements of a claimed invention are set forth in a reference. See, *e.g.*, *Transclean Corp. v. Bridgewood Services, Inc.*, 290 F.3d 1364, 1370 (Fed. Cir. 2002). As such, a reference must clearly disclose each and every limitation of the claimed invention before anticipation may be found. In the instant case, the Examiner has failed to identify the disclosure of each and every limitation of the claimed invention.

For at least these reasons, Appellant respectfully submits that *Mühlbauer*, as evidenced by *Kuttan* and *Wetli*, fails to disclose or suggest each and every element of the present claims.

Accordingly, Appellant respectfully requests that the anticipation rejections of Claims 10, 12-24, 26-28, 38-40 and 45-47 under 35 U.S.C. §102(b) be reconsidered and withdrawn.

VIII. CONCLUSION

Appellant respectfully submits that the Examiner has failed to establish anticipation under 35 U.S.C. §102(b) with respect to the rejections of Claims 10, 12-24, 26-28, 38-40 and 45-47. Accordingly, Appellant respectfully submits that the anticipation rejection is erroneous in law and in fact and should, therefore, be reversed by this Board.

The Director is authorized to charge $540.00 for the Appeal Brief and any additional fees which may be required, or to credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 3717519-00060 on the account statement.

Respectfully submitted,

K&L GATES LLP

BY _____

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Dated: April 20, 2011

CLAIMS APPENDIX

PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 10/580,186

10. A nutritional composition comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof, a nutritionally acceptable carrier, and a fat source.

12. The nutritional composition of Claim 10, wherein the γ-glutamyl-alkenyl-cysteine sulfoxide is γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.

13. The nutritional composition of Claim 10 further comprising:

(a) a calcium source,

(b) at least one energy source selected from the group consisting of a carbohydrate source a nitrogen source, and combinations thereof, and optionally

(c) Vitamin D.

14. The nutritional composition of Claim 13, wherein the calcium source (a) is an organic calcium salt.

15. The nutritional composition of Claim 13, wherein the carbohydrate source of component (b) is selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylitol, sorbitol, and mixtures thereof.

16. The nutritional composition of Claim 10, wherein the fat source of component (b) is selected from the group consisting of omega-6 polyunsaturated fatty acid sources, omega-3 polyunsaturated fatty acid sources, mono-unsaturated fatty acid sources, C_6-C_{12}- fatty acid sources, and mixtures thereof.

17. The nutritional composition of Claim 13, wherein the nitrogen source of component (b) is selected from the group consisting of soy bean derived proteins; milk proteins, protein hydrolysates, a mixture of essential amino acids and arginine, and mixtures thereof.

18. The nutritional composition of Claim 13, wherein the carbohydrate source provides for 30 to 70 %, the nitrogen source for 5 to 40 %, and the fat source for 0.01 to 5 % of the total energy supply of the composition.

19. The nutritional composition of Claim 13 comprising from 3 to 25 % by weight of component (a), from 5 to 50 % by weight of component (b) and from 1 to 95 % by weight of component (c), based on the total weight of the nutritional composition.

20. The nutritional composition of Claim 10 further comprising 0.2 to 10 % by weight of other nutritionally acceptable components chosen from vitamins, minerals, trace elements, fibers, flavors, preservatives, colorants, sweeteners and emulsifiers.

21. The nutritional composition of Claim 10 in the form of a dietary supplement providing from 50 to 1500 kcal/day, or in the form of an animal feed supplement.

22. The nutritional composition of Claim 10 in liquid form.

23. The nutritional composition of Claim 10 in granulate or powder form.

24. A pharmaceutical composition in single unit dose form, comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamy-alkenyl-cysteine sulfoxide, and combinations thereof, a pharmaceutically acceptable carrier, and a fat source.

26. The pharmaceutical composition of Claim 24, wherein the γ-glutamyl-alkenyl-cysteine sulfoxide is γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide.

27. The pharmaceutical composition of Claim 24 for enteral administration in the form of a dragée, tablet, capsule, sachet or suppository.

28. The pharmaceutical composition of Claim 24 in the form of a veterinary composition.

38. The nutritional composition as claimed in Claim 10, wherein γ-glutamyl-peptide inhibits dose-dependently the resorption activity of osteoclasts.

39. The nutritional composition as claimed in Claim 10, wherein the minimal effective dose is about 2 mM.

40. The nutritional composition as claimed in Claim 10, wherein the dose is at least 2 mM.

45. The pharmaceutical composition of Claim 24, wherein γ-glutamyl-peptide inhibits dose-dependently the resorption activity of osteoclasts.

46. The pharmaceutical composition of Claim 24, wherein the minimal effective dose is about 2 mM.

47. The pharmaceutical composition of Claim 24, wherein the dose is at least 2 mM.

EVIDENCE APPENDIX

EXHIBIT A: Non-final Office Action dated July 16, 2010

EXHIBIT B: Final Office Action dated November 24, 2010

EXHIBIT C: Advisory Action dated February 4, 2011

EXHIBIT D: WO 98/50054 to Mühlbauer ("*Mühlbauer*")

EXHIBIT E: Kuttan et al. ("*Kuttan*")

EXHIBIT F: J. Agric. Food Chem., 2005, 53(9): 3408-3014 to Wetli et al. ("*Wetli*")

RELATED PROCEEDINGS APPENDIX

None.

EXHIBIT A

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/580,186	09/21/2007	Rudolf Brenneisen	8588-US	3801

74476 7590 07/16/2010

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EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
07/16/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdepartment@rd.nestle.com
athena.pretory@rd.nestle.com

Office Action Summary	Application No.	Applicant(s)	
	10/580,186	BRENNEISEN ET AL.	
	Examiner	Art Unit	
	JULIE HA	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *07 May 2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-10,12-24,26-33 and 36-47* is/are pending in the application.

4a) Of the above claim(s) *1-9,29-33,36,37 and 41-44* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *10, 12-24, 26-28, 38-40 and 45-47* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Amendment after Non-final office action filed on May 07, 2010 is acknowledged. Claims 11, 25, 34-35 have been cancelled. Claims 1-10, 12-24, 26-33 and 36-47 are pending in this application. Applicant elected with traverse of Group 3 and the election of species γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide as the γ-glutamyl peptide, skim milk powder as the calcium source, maltodextrins as the carbohydrate, omega-6 polyunsaturated fatty acid source as the fat source, soy bean derived protein as the nitrogen source, Vitamin A as the vitamin, potassium as the mineral, gum Arabic as the fiber, vegetable flavors as the flavor, and Allium cepa as the Allium, and further elected γ-glutamyl-alkyl-cysteine sulfoxide as the γ-glutamyl-peptide, osteoporosis as the disease, calcium chloride as the calcium source, carbohydrate as the energy source, maltodextrins as the carbohydrate, vitamin D as the vitamin on November 02, 2009. The traversal was not found persuasive, and the restriction was deemed proper and was made FINAL in the previous office action. There were inconsistencies between the elected species filed on August 11, 2009 and November 02, 2009. For the purpose of this examination, the election of species filed on November 02, 2009 was examined. Search was conducted on the elected species, and prior art was found. A prior art WO 98/50054 A1 teaches the other nonelected species. Therefore, election of species was withdrawn in the previous office action. Claims 1-9, 33, 36-37 and 41-44 are withdrawn from further consideration, pursuant to 37 CFR 1.142(b), as being drawn to nonelected inventions, there being no allowable generic or linking claim. Claims 29-32, previously drawn to a g-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide by fractionation of an

hydrophilic, ethanolic extract of Allium have been amended to method claims.

Therefore, Claims 29-32 are hereby withdrawn from consideration, as being drawn to nonelected invention. Claims 10, 12-24, 26-28, 38-40 and 45-47 are examined on the merits in this office action.

After further review, a non-final rejection follows below.

Withdrawn Objection and Rejections

1. Objection to claims 34-35 as being improper dependent form for failing to further limit the subject matter of a previous claim is hereby withdrawn in view of Applicant's cancellation of claims 34-35.

2. Rejection of claims 10, 23-24, 27 and 45 under 35 U.S.C. 102(b) as being anticipated by Blatt et al (US Patent No. 6,270,803), is hereby withdrawn in view of Applicant's amendment to the claims.

3. Rejection of claims 10-12, 22-26, 29-32, 34-35, 38 and 45 under 35 U.S.C. 102(b) as being anticipated by Kuttan et al (Biochemistry, 1974, 13(21): 4394-4400, filed with IDS), is hereby withdrawn in view of Applicant's amendment to the claims.

4. Rejection of claims 10-32, 34-35, 38-40, and 45-47 under 35 U.S.C. 102(b) as being anticipated by Muhlbauer (WO 98/50054, filed with IDS), is hereby withdrawn in view of Applicant's amendment to the claims.

New Rejection

35 U.S.C. 102

5. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

6. Claims 10, 12-24, 26-28, 38-40 and 45-47 are rejected under 35 U.S.C. 102(b) as being anticipated by Muhlbauer (WO 98/50054, filed with IDS) as being evidenced by Kuttan et al (Biochemistry, 1974, 13(21): 4394-4400, filed with IDS) and as evidenced by Wetli et al (J. Agric. Food Chem., 2005, 53(9): 3408-3014, abstract only provided. Full reference requested).

7. Muhlbauer reference teaches a nutritional composition comprising all of the active components of instant claims (see throughout the reference, Claims 5-20), meeting the limitation of instant claims 10 in part, 38 and 45. The reference teaches that the nutritional or pharmaceutical compositions containing a plant extract or concentrate selected from the group consisting of allium, eruca, petroselinum and brassica extracts or concentrates (see abstract and p. 2, last paragraph). The reference teaches that the composition is useful for the treatment of diseases or conditions which are characterized by increased bone resorption, osteoporosis (see abstract). The reference teaches that the term allium refers to the genus allium and includes for example any member of the botanical species Allium cepa (onion), Allium ascalonium and so on, and

indicates that the preferred extract is from Allium cepa (see p. 3, 2nd paragraph, see p. 4, 6th paragraph). The onion extracts and concentrates are prepared from the whole eatable part of the vegetable (see p. 3, 3rd paragraph). The reference teaches that the extract and concentrates of the above-mentioned plants or vegetables may be in liquid form or in solid form such as in granulate or powder form (see p. 5, 1st paragraph), meeting the limitation of claims 22-23. The reference teaches that suitable methods of obtaining extracts of the above-mentioned plants or vegetables are known in the art…by extracting the fresh cut or dried plants or vegetables or the respective roots, fruits or seeds thereof for example with water or with one or more food grade solvents or with a mixture of water and one or more food grade solvents…ethanol (see p. 5, 3rd paragraph). Further, Example 4 at page 16, explicitly teaches ethanol/water extraction. As evidenced by Kuttan et al, γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide isolated from sandal (Santalum album L.) is the same as the protein isolated from onion (Allium cepa) (see abstract). The reference teaches that γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide is in aqueous solutions, water (see p. 4396, right column, "CD Absorption"). Therefore, the ethanolic extract of allium cepa of the reference would inherently comprise the γ-L-glutamyl-trans-S-l-propenyl-L-cysteine sulfoxide of the instant claims. The reference teaches that the extract may be used in liquid form, particularly in aqueous form, or in solid form, granulate or powder form. If the extracts in liquid form, it has a solid contents of for example from 1 to 25% by weight, preferably from 2 to 20% by weight and most preferred from 2 to 15% by weight (see p. 6, 2nd paragraph).

The reference teaches that the subject to be treated is an adult person a satisfactory inhibitory effect on bone resorption is, in general obtained with compositions formulated to allow a daily administration of 0.1 to 20 grams, preferably 0.2 to 15 grams and most preferred 0.4 to 10 grams of allium, petroselinum, brassica and/or eruca concentrate or extract (see p. 6, 2nd paragraph). The reference further teaches that suitable nutritional compositions comprising the plant/vegetable extracts comprise at least one (a) plant/vegetable extract or concentrate from allium, (b) a calcium source, and (c) at least one energy source selected from carbohydrate, fat and nitrogen sources, and Vitamin D (see p. 6, last paragraph, claim 5), meeting the limitation of instant claims 10, 12, 38 and 45. Since the nutritional composition comprises the same active compound, this would inherently have the same functionality and characteristics of instant claims 38 and 45. The reference teaches that from approximately 0.1 to 40%, preferably from approximately 3 to 25% of plant/vegetable extract or concentrate component (a) (see p. 6, last paragraph); calcium source such as calcium chloride or skim milk and the calcium source (b) is in one unit dosage from about 100 mg to 1000 mg, preferably 200 mg to 700 mg or from approximately 1 to 60 %, preferably from approximately 5 to 50% of calcium component (b) (see p. 7, 1st and 2nd paragraph); suitable carbohydrate sources include for example maltodextrins, starch, lactose, glucose (see p. 7, 3rd paragraph); suitable fat sources include omega-6 polyunsaturated fatty acid (see p. 7, 4th paragraph); suitable nitrogen sources such as soybean derived proteins (see p. 8, 4th paragraph), meeting the limitation of claims 14-17 and 19. The reference teaches that the nutritional composition comprise from approximately 0.1 % to

98.9%, preferably from approximately 1 to approximately 95% of energy source (p. 9, 1st paragraph), further meeting the limitation of claim 19. The reference teaches that the carbohydrate source provides for 30 to 70% of the total energy supply, the nitrogen source for 5 to 45 %, and the fat source for 0.1 to 15% of the total energy supply (see p. 9, 2nd paragraph), meeting the limitation of instant claim 18. Further, the reference teaches that the nutritional formulation may comprise other nutritionally acceptable components such as vitamins (see p. 10, 1st paragraph), meeting the limitation of instant claim 20. The reference teaches that the supplement comprises energy sources in an amount supplying from 50 to 1500 kcal/day (see p. 11, 2nd paragraph, see claim 16), meeting the limitation of instant claim 21. The reference teaches that the nutritional formulation is formulated in any form suitable for enteral administration, in aqueous form or in powder or granulate form, whereby the powder or granulate is conveniently added to water prior to use (see p. 11, 1st and 2nd paragraphs), meeting the limitation of claims 24 and 26. Additionally, the reference teaches dragee, table, capsule, sachet or suppository compositions (see p. 12, 3rd paragraph, see claim 20), meeting the limitation of instant claims 27-28.

Furthermore, the reference teaches that 250 mg freeze-dried onion extract are obtained for each g of dry whole onion, and the onion extract (0.017, 0.17, 1.7 mg onion extract/ ml medium) inhibited osteoclast-mediated resorption (see column 12, lines 1-9). As evidenced by Wetli et al, the molecular mass of gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide is 306 Da (see abstract). The onion extract at 0.017 mg/ ml would yield 55.5 μM effective dose; at 0.17 mg/ml would yield 555.5 μM effective

dose; at 1.7 mg/ml would yield 5.55 mM effective dose, Meeting the limitation of instant claims 39-40 and 46-47. Therefore, the reference anticipates instant claims 10, 12-24, 26-28, 38-40 and 45-47.

Conclusion

8. No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JULIE HA whose telephone number is (571)272-5982. The examiner can normally be reached on Mon-Thurs, 5:30 AM to 4:00 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Cecilia Tsang can be reached on 571-272-0562. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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/Julie Ha/
Examiner, Art Unit 1654

Notice of References Cited	Application/Control No. 10/580,186	Applicant(s)/Patent Under Reexamination BRENNEISEN ET AL.	
	Examiner JULIE HA	Art Unit 1654	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

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	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Wetli HA, Brenneisen R, Tschudi I, Langos M, Bigler P, Sprang T, Schurch S, Muhlbauer RC, "A gamma-glutamyl peptide isolated from onion (ALlium cepa L.) by bioassay-guided fractionation inhibits resorption activity of osteoclasts," J. Agric Food Chem, 2005, 53(9): 3408-3014. Abstract only provided.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EXHIBIT B

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/580,186	09/21/2007	Rudolf Brenneisen	8588-US	3801

74476 7590 11/24/2010

Nestle HealthCare Nutrition
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EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
11/24/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdepartment@rd.nestle.com
athena.pretory@rd.nestle.com

Office Action Summary	Application No.	Applicant(s)	
	10/580,186	BRENNEISEN ET AL.	
	Examiner	Art Unit	
	JULIE HA	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *15 September 2010*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-10,12-24,26-33 and 36-47* is/are pending in the application.

4a) Of the above claim(s) *1-9,29-33,36,37 and 41-44* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *10,12-24,26-28,38-40 and 45-47* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Response after Non-final office action filed on September 15, 2010 is acknowledged. Claims 1-10, 12-24, 26-33 and 36-47 are pending in this application. Applicant elected with traverse of Group 3 and the election of species γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide as the γ-glutamyl peptide, skim milk powder as the calcium source, maltodextrins as the carbohydrate, omega-6 polyunsaturated fatty acid source as the fat source, soy bean derived protein as the nitrogen source, Vitamin A as the vitamin, potassium as the mineral, gum Arabic as the fiber, vegetable flavors as the flavor, and Allium cepa as the Allium, and further elected γ-glutamyl-alkyl-cysteine sulfoxide as the γ-glutamyl-peptide, osteoporosis as the disease, calcium chloride as the calcium source, carbohydrate as the energy source, maltodextrins as the carbohydrate, vitamin D as the vitamin on November 02, 2009. The traversal was not found persuasive, and the restriction was deemed proper and was made FINAL in the previous office action. There were inconsistencies between the elected species filed on August 11, 2009 and November 02, 2009. For the purpose of this examination, the election of species filed on November 02, 2009 was examined. Search was conducted on the elected species, and prior art was found. A prior art WO 98/50054 A1 teaches the other nonelected species. Therefore, election of species was withdrawn in the previous office action. Claims 1-9, 33, 36-37 and 41-44 are withdrawn from further consideration, pursuant to 37 CFR 1.142(b), as being drawn to nonelected inventions, there being no allowable generic or linking claim. Claims 29-32, previously drawn to a γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide by fractionation of an hydrophilic, ethanolic extract of Allium have

been amended to method claims. Therefore, Claims 1-9, 29-33, 36-37 and 41-44 remain withdrawn from consideration, as being drawn to nonelected invention. Claims 10, 12-24, 26-28, 38-40 and 45-47 are examined on the merits in this office action.

1. This application contains claims 1-9, 29-33, 36-37 and 41-44 drawn to an invention nonelected with traverse in the reply filed on November 02, 2009. A complete reply to the final rejection must include cancellation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

Maintained Rejection

35 U.S.C. 102

2. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

3. Claims 10, 12-24, 26-28, 38-40 and 45-47 remain rejected under 35 U.S.C. 102(b) as being anticipated by Muhlbauer (WO 98/50054, filed with IDS) as being evidenced by Kuttan et al (Biochemistry, 1974, 13(21): 4394-4400, filed with IDS) and as evidenced by Wetli et al (J. Agric. Food Chem., 2005, 53(9): 3408-3014, abstract only provided in the previous office action and full article provided herein).

4. Muhlbauer reference teaches a nutritional composition comprising all of the active components of instant claims (see throughout the reference, Claims 5-20), meeting the limitation of instant claims 10 in part, 38 and 45. The reference teaches that the nutritional or pharmaceutical compositions containing a plant extract or concentrate selected from the group consisting of allium, eruca, petroselinum and brassica extracts or concentrates (see abstract and p. 2, last paragraph). The reference teaches that the composition is useful for the treatment of diseases or conditions which are characterized by increased bone resorption, osteoporosis (see abstract). The reference teaches that the term allium refers to the genus allium and includes for example any member of the botanical species Allium cepa (onion), Allium ascalonium and so on, and indicates that the preferred extract is from Allium cepa (see p. 3, 2nd paragraph, see p. 4, 6th paragraph). The onion extracts and concentrates are prepared from the whole eatable part of the vegetable (see p. 3, 3rd paragraph). The reference teaches that the extract and concentrates of the above-mentioned plants or vegetables may be in liquid form or in solid form such as in granulate or powder form (see p. 5, 1st paragraph), meeting the limitation of claims 22-23. The reference teaches that suitable methods of obtaining extracts of the above-mentioned plants or vegetables are known in the art...by extracting the fresh cut or dried plants or vegetables or the respective roots, fruits or seeds thereof for example with water or with one or more food grade solvents or with a mixture of water and one or more food grade solvents...ethanol (see p. 5, 3rd paragraph). Further, Example 4 at page 16, explicitly teaches ethanol/water extraction. As evidenced by Kuttan et al, γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide

isolated from sandal (Santalum album L.) is the same as the protein isolated from onion (Allium cepa) (see abstract). The reference teaches that γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide is in aqueous solutions, water (see p. 4396, right column, "CD Absorption"). Therefore, the ethanolic extract of allium cepa of the reference would inherently comprise the γ-L-glutamyl-trans-S-l-propenyl-L-cysteine sulfoxide of the instant claims. The reference teaches that the extract may be used in liquid form, particularly in aqueous form, or in solid form, granulate or powder form. If the extracts in liquid form, it has a solid contents of for example from 1 to 25% by weight, preferably from 2 to 20% by weight and most preferred from 2 to 15% by weight (see p. 6, 2nd paragraph).

The reference teaches that the subject to be treated is an adult person a satisfactory inhibitory effect on bone resorption is, in general obtained with compositions formulated to allow a daily administration of 0.1 to 20 grams, preferably 0.2 to 15 grams and most preferred 0.4 to 10 grams of allium, petroselinum, brassica and/or eruca concentrate or extract (see p. 6, 2nd paragraph). The reference further teaches that suitable nutritional compositions comprising the plant/vegetable extracts comprise at least one (a) plant/vegetable extract or concentrate from allium, (b) a calcium source, and (c) at least one energy source selected from carbohydrate, fat and nitrogen sources, and Vitamin D (see p. 6, last paragraph, claim 5), meeting the limitation of instant claims 10, 12, 38 and 45. Since the nutritional composition comprises the same active compound, this would inherently have the same functionality and characteristics of instant claims 38 and 45. The reference teaches that from approximately 0.1 to 40%,

preferably from approximately 3 to 25% of plant/vegetable extract or concentrate component (a) (see p. 6, last paragraph); calcium source such as calcium chloride or skim milk and the calcium source (b) is in one unit dosage from about 100 mg to 1000 mg, preferably 200 mg to 700 mg or from approximately 1 to 60 %, preferably from approximately 5 to 50% of calcium component (b) (see p. 7, 1st and 2nd paragraph); suitable carbohydrate sources include for example maltodextrins, starch, lactose, glucose (see p. 7, 3rd paragraph); suitable fat sources include omega-6 polyunsaturated fatty acid (see p. 7, 4th paragraph); suitable nitrogen sources such as soybean derived proteins (see p. 8, 4th paragraph), meeting the limitation of claims 14-17 and 19. The reference teaches that the nutritional composition comprise from approximately 0.1 % to 98.9%, preferably from approximately 1 to approximately 95% of energy source (p. 9, 1st paragraph), further meeting the limitation of claim 19. The reference teaches that the carbohydrate source provides for 30 to 70% of the total energy supply, the nitrogen source for 5 to 45 %, and the fat source for 0.1 to 15% of the total energy supply (see p. 9, 2nd paragraph), meeting the limitation of instant claim 18. Further, the reference teaches that the nutritional formulation may comprise other nutritionally acceptable components such as vitamins (see p. 10, 1st paragraph), meeting the limitation of instant claim 20. The reference teaches that the supplement comprises energy sources in an amount supplying from 50 to 1500 kcal/day (see p. 11, 2nd paragraph, see claim 16), meeting the limitation of instant claim 21. The reference teaches that the nutritional formulation is formulated in any form suitable for enteral administration, in aqueous form or in powder or granulate form, whereby the powder or granulate is conveniently added

to water prior to use (see p. 11, 1st and 2nd paragraphs), meeting the limitation of claims 24 and 26. Additionally, the reference teaches dragee, table, capsule, sachet or suppository compositions (see p. 12, 3rd paragraph, see claim 20), meeting the limitation of instant claims 27-28.

Furthermore, the reference teaches that 250 mg freeze-dried onion extract are obtained for each g of dry whole onion, and the onion extract (0.017, 0.17, 1.7 mg onion extract/ ml medium) inhibited osteoclast-mediated resorption (see column 12, lines 1-9). As evidenced by Wetli et al, the molecular mass of gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide is 306 Da (see abstract). The onion extract at 0.017 mg/ ml would yield 55.5 μM effective dose; at 0.17 mg/ml would yield 555.5 μM effective dose; at 1.7 mg/ml would yield 5.55 mM effective dose, Meeting the limitation of instant claims 39-40 and 46-47. Therefore, the reference anticipates instant claims 10, 12-24, 26-28, 38-40 and 45-47.

Response to Applicant's Arguments

5. Applicant argues that "Applicant has surprisingly found that the active constituent of allium responsible for the bone resorption inhibiting effect may be found in a hydrophilic, ethanolic extract of allium such as allium cepa." Applicant argues that "Muhlbauer fails to disclose or suggest nutritional and pharmaceutical compositions, respectively, comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ -glutamyl-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier, and a fat source as required, in part, by independent claims 10 and 24. Instead Muhlbauer is entirely directed to plant extracts for the

treatment of increase bone resorption." Applicant further argues that "Muhlbauer also fails to disclose or suggest a method of obtaining a γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide by fractionation of an hydrophilic, ethanolic extract of Allium, the method comprising the steps of obtaining an hydrophilic, ethanolic extract of Allium cepa, separating saccharides from fraction A, further separating saccharides from fraction A1, and further frantionation by semi-preparative reversed-phase HPLC (SP-RP-HPLC)." Applicant further argues that "Kuttan and Weil fail to disclose or suggest nutritional and pharmaceutical compositions, respectively, comprising a γ-glutamyl-peptide selected from the group consisting of γ-glutamyl-alkyl-cysteine sulfoxide, γ-glutamyl-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier, and a fat source, in part, by independent claims 10 and 24. Kuttan and Weil also fail to disclose or suggest method of obtaining a γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide by fractionation of an hydrophilic, ethanolic extract of Allium, the method comprising the steps of obtaining an hydrophilic, ethanolic extract of Allium cepa, separating saccharides from fraction A, further separating saccharides from fraction A1, and further frantionation by semi-preparative reversed-phase HPLC (SP-RP-HPLC)."

6. Applicant's arguments have been fully considered but have not been found persuasive. For the record, Wetli reference was used, and not Weil reference as in Applicant's remarks. The reference teaches all of the active components of instant claims. Muhlbauer reference teaches that the nutritional or pharmaceutical compositions containing a plant extract or concentrate selected from the group consisting of **allium**, eruca, petroselinum and brassica extracts or concentrates. The reference teaches that

the composition is useful for the treatment of diseases or conditions which are characterized by increased bone resorption, osteoporosis. The reference teaches that the term **allium** refers to the genus allium and includes for example any member of the botanical species **Allium cepa (onion), Allium ascalonium** and so on, and indicates that the preferred extract is from **Allium cepa**. The reference teaches that the extract and concentrates of the above-mentioned plants or vegetables may be in liquid form or in solid form such as in granulate or powder. The reference teaches that suitable methods of obtaining extracts of the above-mentioned plants or vegetables are known in the art...by extracting the fresh cut or dried plants or vegetables or the respective roots, fruits or seeds thereof for example **with water or with one or more food grade solvents or with a mixture of water and one or more food grade solvents...ethanol**. Example 4 at page 16, explicitly teaches ethanol/water extraction. The instant specification discloses that "The active constituent of allium responsible for the bone resorption inhibiting effect, may be found in an hydrophilic, ethanolic extract of allium such as Allium cepa" (see paragraph [0012] of instant specification US 2008/0194492). Both Kuttan and Wetli references were provided as evidence to show that γ-glutamyl peptide is isolated from Allium cepa. Kuttan et al teach that γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide isolated from sandal (Santalum album L.) and this is the same as the protein isolated from onion (Allium cepa) (see abstract). The reference teaches that γ-L-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide is in aqueous solutions, water (see p. 4396, right column, "CD Absorption"). The reference teaches the same water (hydrophilic)/ethanolic extract as the instant specification.

Therefore, the water/ethanolic extract of allium cepa of the reference would inherently comprise the γ-L-glutamyl-trans-S-I-propenyl-L-cysteine sulfoxide of the instant claims. Therefore, the reference anticipates instant claims 10, 12-24, 26-28, 38-40 and 45-47.

In regards Applicant's argument that "Muhlbauer, Kuttan and Weil fail to disclose or suggest to a method of obtaining a γ-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide by fractionation of an hydrophilic, ethanolic extract of Allium, the method comprising the steps of obtaining an hydrophilic, ethanolic extract of Allium cepa, separating saccharides from fraction A, further separating saccharides from fraction A1, and further frantionation by semi-preparative reversed-phase HPLC (SP-RP-HPLC)" the claims drawn to the method claims (claims 29-33, 36-37, 41-44) have been withdrawn from further consideration, as being drawn to nonelected elections. Therefore, these claims were not under examination, and thus, the argument is moot.

Conclusion

7. No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JULIE HA whose telephone number is (571)272-5982. The examiner can normally be reached on Mon-Thurs, 5:30 AM to 4:00 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Cecilia Tsang can be reached on 571-272-0562. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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/Julie Ha/
Primary Examiner, Art Unit 1654

Notice of References Cited	Application/Control No. 10/580,186	Applicant(s)/Patent Under Reexamination BRENNEISEN ET AL.	
	Examiner JULIE HA	Art Unit 1654	Page 1 of 1

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FOREIGN PATENT DOCUMENTS

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	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EXHIBIT C

UNITED STATES PATENT AND TRADEMARK OFFICE

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EXAMINER	
HA, JULIE	

ART UNIT	PAPER NUMBER
1654	

NOTIFICATION DATE	DELIVERY MODE
02/04/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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Advisory Action Before the Filing of an Appeal Brief	**Application No.** 10/580,186	**Applicant(s)** BRENNEISEN ET AL.	
	Examiner JULIE HA	**Art Unit** 1654	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 20 January 2011 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:
   a) ☐ The period for reply expires _____months from the mailing date of the final rejection.
   b) ☒ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

   Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☐ The Notice of Appeal was filed on _____. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☐ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   (a) ☐ They raise new issues that would require further consideration and/or search (see NOTE below);
   (b) ☐ They raise the issue of new matter (see NOTE below);
   (c) ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   (d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): a) ☐ will not be entered, or b) ☒ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.
   The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: _____.
   Claim(s) objected to: _____.
   Claim(s) rejected: *10,12-24,26-28,38-40 and 45-47*.
   Claim(s) withdrawn from consideration: *1-9,29-33,36,37 and 41-44*.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: Please see continuation of 11 below.
12. ☐ Note the attached Information *Disclosure Statement*(s). (PTO/SB/08) Paper No(s). _____
13. ☐ Other: _____.

/Julie Ha/
Primary Examiner, Art Unit 1654

Continuation of 11:

Claims 10, 12-24, 26-28, 38-40 and 45-47 remain rejected under 35 U.S.C. 102(b) as being anticipated by Muhlbauer (WO 98/50054) as being evidenced by Kuttan et al (Biochemistry, 1974, 13(21): 4394-4400) and as evidenced by Wetli et al (J. Agric. Food Chem., 2005, 53(9): 3408-3414), as set forth in the previous office action.

Applicant argues that "Independent claims 10 and 24 recite, in part, nutritional and pharmaceutical compositions, respectively, comprising a g-glutamyl-peptide selected from the group consisting of g-glutamyl-alkyl-cysteine sulfoxide, g-glutamyl-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier and a fat source. Independent claim 29 recites, in part, a method of obtaining a g-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide by fractionaation of an hydrophilic ethanolic extract of Allium..." Applicant argues that "Applicant has surprisingly found that the active constituent of allium responsible for the bone resorption inhibiting effect may be found in a hydropholic, ethanolic extract of allium such as allium cepa." Applicant argues that "Muhlbauer fails to disclose or suggest nutritional and pharmaceutical compositions, respectively, comprising a g-glutamyl-peptide selected from the group consisting of g-glutamyl-alkyl-cysteine sulfoxide, g-glutamyl-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier and a fat source." Applicant further argues that "Kuttan and Wetli fail to disclose or suggest nutrional and pharmaceutical compositions, respectively, comprising a g-glutamyl-peptide selected from the group consisting of g-glutamyl-alkyl-cysteine sulfoxide, g-glutamyl-alkenyl-cysteine sulfoxide, and combinations thereof, a carrier and a fat source.

Applicant's arguments have been fully considered but have not been found persuasive. Muhlbauer reference teaches that the nutritional or pharmaceutical compositions containing a plant extract or concentrate selected from the group consisting of allium, eruca, petroselinum and brassica extracts or concentrates.Muhlbauer further teaches that the composition is useful for the treatment of diseases or conditions which are characterized by increased bone resorption, osteoporosis. The reference teaches that the term allium refers to the genus allium and includes any member of the botanical spceis Allium cepa (onion), Allium ascalonium and so on. The reference teaches that the concentrate or plant extract is obtained by extracting the fresh cut or dried plants or vegetables or the respective roots, fruits, seeds thereof with water or with one or more food grade solvents or with a mixture of water and one or more food grade solvents, ethanol. Example 4 at page 16 explicitly teaches ethanol/water extraction. The instant specification discloses that "The active constituent of allium responsivle for the bone resorption inhibiting effect, may be found in an hydrophilic, ethanolic extract of allium such as Allium cepa" (see paragraph [0012]). Muhlbauer reference further teaches that the nutritional composition comprise at least one (a) plant/extract or concentrate from allium, (b) a calcium source, and (c) at least one energy source selected from carbohydrate, fat and nitrogen sources, and Vitamin C.
Both Kuttan and Wetli references were provided as evidence to show that g-glutamyl peptide is isolated from Allium cepa. Kuttan et al teach that g-glutamyl-S-(trans-l-propenyl)-L-cysteine sulfoxide isolated from sandal (Santalum album L.) and that is the same as the protein isolated from onion (Allium cepa).
In regards to Applicant's argument regarding claim 29 (claims 29-33, 36-37, 41-44), these claims are drawn to the method claims. These claims have been withdrawn from further consideration, as being drawn to nonelected inventions. Therefore, these claims were not under examination, and thus, the argument is moot.

EXHIBIT D

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(54) Title: PLANT EXTRACTS FOR THE TREATMENT OF INCREASE BONE RESORPTION

(57) Abstract

The present invention is concerned with nutritional or pharmaceutical compositions containing a plant extract or concentrate selected from the group consisting of allium, eruca, petroselinum and brassica extracts and concentrates or mixtures thereof. The compositions of the invention are useful for the treatment or prophylaxis of diseases or conditions which are characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis.

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Plant Extracts for the Treatment of Increased Bone Resorption

The present invention relates to nutritional or pharmaceutical compositions comprising extracts or concentrates of certain plants and their use as inhibitors of bone resorption.

The most common metabolic bone disorder is osteoporosis. Osteoporosis can be generally defined as the reduction in the quantity of bone, either from the reduction in bone formation or the acceleration of bone resorption, in either event the result is a decrease in the amount of skeletal tissue. Osteoclasts (bone resorbing cells) are responsible for the excavation of a portion of bone during the resorption process. After resorption, osteoblasts (bone forming cells) appear, which then refill the resorbed portion with new bone.

In young healthy adults, the rate at which the osteoclasts and osteoblasts are formed and operate maintains a balance between bone resorption and bone formation. However, as normal consequence of aging, an imbalance in this remodeling process develops, resulting in loss of bone. As imbalance continues over time, the reduction in bone mass and thus bone strength leads to fractures.

Many compositions and methods are described in the medical literature for the treatment of osteoporosis. For example, estrogens, calcitonin and bisphosphonates are known to be effective inhibitors of bone resorption.

It has now surprisingly been found that products derived from certain plants or vegetables which belong for example to the botanical families of liliaceae, umbelliferae and cruciferae have a potent inhibitory effect on bone resorption.

Accordingly, the present invention relates to the use of a vegetable extract or concentrate, excluding extracts or concentrates derived from leguminosae and hop, having an inhibitory effect on bone resorption in the preparation of a medicament or nutritional formulation for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis.

By the term leguminosae is meant the botanical family leguminosae (pea family) which includes for example soybean, beans, chick pea or lentil. By hop is meant the botanical species Humulus lupulus.

Osteoporosis as used herein includes osteoporosis induced by hormone deficiency (e.g. postmenopausal) and old age, as well as secondary osteoporosis such as osteoporosis secondary to steroid treatment or secondary to malnutrition caused by anorexia nervosa.

The invention further provides a method for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis, comprising the administration of a medicament or nutritional formulation to a human or other mammal, said medicament or nutritional formulation comprising a vegetable extract or concentrate, excluding extracts or concentrates derived from leguminosae and hop, in an amount which is effective for inhibiting bone resorption.

The present invention also foresees the use of an extract or concentrate from a plant selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates in the preparation of a medicament or nutritional formulation for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis.

Also provided is a method for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis, comprising the administration of a medicament or nutritional formulation to a human or other mammal, said medicament or nutritional formulation comprising an extract or concentrate from a plant selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates, in an amount which is effective for inhibiting bone resorption.

Preferably the extracts and concentrates from allium, petroselinum, brassica and/or eruca are vegetable extracts or concentrates.

As used herein, the term vegetable refers to a herbaceous plant which has an edible portion which is consumed by humans in either raw or cooked form. The edible portion may be a root, such as rutabaga, beet, carrot, and sweet potato; a tuber or storage stem, such as potato and taro; the stem, as in asparagus and kohlrabi; a bud, such as brussels sprouts; a bulb, such as onion and garlic; a petiole or leafstalk, such as celery and rhubarb; a leaf, such as cabbage, lettuce, parsley and spinach; an immature flower, such as cauliflower, broccoli and artichoke; a seed; the immature fruit, such as eggplant, cucumber, and sweet corn (maize); or the mature fruit, such as tomato and pepper.

As used herein, the term allium refers to the genus allium (latin for garlic, a member of the onion family) and includes for example any member of the botanical species *Allium cepa* (onion), *Allium ascalonicum* (shallot), *Allium ampeloprasum* (leek/great-headed-garlic), *Allium porrum* (leek), *Allium schoenoprasum* (chive), *Allium ursinum* (bear's garlic), *Allium sativum* (garlic) or *Allium fistulosum* (bunching onion). Preferred species are *Allium ascalonicum* (shallot), *Allium porrum* (leek), *Allium cepa* (onion) and *Allium ursinum* (bear's garlic, also known as bear paw garlic), particularly the latter two, whereby *Allium cepa* is particularly preferred. Examples of members of the species *Allium cepa* are common onions (with red or white or yellow skins) or shallots, whereby red or white common onions are preferred.

The onion extracts and concentrates are prepared e.g. from the whole eatable part of the vegetable. Suitable chive extracts and concentrates are obtained e.g. from chive herbs. Suitable bear's garlic extracts and concentrates are obtained e.g. from bear's garlic bulbs, fresh herbs or from the whole blooming plant, preferably they are obtained form fresh herbs.

The term petroselinum as used herein refers to the genus petroselinum (common name parsley) and includes for example any member of the botanical species *Petroselinum crispum*. Examples are *Petroselinum crispum crispum*, that is common parsley with curly leaves, *Petroselinum crispum radiosum* or *Petroselinum crispum var. neapolitanum* also known as Italian Parsley with flat leaves.

Suitable petroselinum extracts or concentrates may be produced e.g. from roots, fruits or seeds, or particularly from herbs.

As used herein the term brassica refers to the genus brassica (latin for cabbage) and includes for example any member of the botanical species *Brassica oleracea*, *Brassica napus*, *Brassica rapa*, *Brassica alboglabra*, *Brassica juncea*, *Brassica perviridis*, *Brassica alba* and *Brassica nigra*.

Brassica oleracea is a preferred species, particularly preferred members of this species are *Brassica oleracea var. italica*, i.e. broccoli, or *Brassica oleracea var. gemmifer*a, i.e. Brussels sprouts. Broccoli extracts and concentrates are particularly preferred as brassica extract or concentrate. Suitable extracts and concentrates of brassica oleraceae species are produced advantageously from the whole eatable part of the vegetable or from the freshly germinated sprouts or shoots.

As used herein the term eruca refers to the genus eruca and includes in particular any member of the botanical species *Eruca sativa* (wild form) or *Eruca vesicaria* subsp. *sativa* (cultivated form) for which the common name is arrugula or roquette.

The plant/vegetable extracts and concentrates of the invention are preferably obtained from an edible portion of the plant or vegetable. By edible portion is meant the portion which is consumed by humans in either raw or cooked form.

A preferred group of inventive plant/vegetable extracts and concentrates comprises concentrates or extracts from any member of the botanical species *Allium cepa*, *Allium ascalonicum*, *Allium ursinum*, *Petroselinum crispum*, *Brassica oleracea* or *Eruca sativa*. A more preferred group of inventive plant/vegetable extracts and concentrates comprises extracts and concentrates from any member of the botanical species *Allium cepa*, *Petroselinum crispum* (in particular *Petroselinum crispum crispum* and *Petroselinum crispum var. neapolitanum*) and *Brassica oleracea* (in particular *Brassica oleracea var. italica*), particularly extracts and concentrates of onions (*Allium cepa*), Italian Parsley (*Petroselinum crispum var. neapolitanium*) or broccoli (*Brassica oleracea var. italica*). The use of onion extracts (in particular white onion extracts) is particularly preferred.

The extracts and concentrates of the above-mentioned plants or vegetables may be in liquid form or in solid form such as in granulate or powder form.

Suitable plant or vegetable concentrates are obtainable e.g. by drying or freeze-drying the fresh-cut plants or vegetables or the respective roots, fruits or seeds thereof and then optionally grinding or granulating the dried material; or by squeezing the fresh-cut plants or vegetables or the respective roots, fruits or seeds thereof and gathering the liquid fraction and optionally drying it. The use of a concentrate of the above-mentioned plants or vegetables in solid form and particularly in powder form is preferred.

Suitable methods of obtaining extracts of the above-mentioned plants or vegetables are known in the art. The plant or vegetable extracts are obtainable e.g. by extracting the fresh-cut or dried plants or vegetables or the respective roots, fruits or seeds thereof for example with water or with one or more food grade solvents or with a mixture of water and one or more food grade solvents. Suitable food grade solvents include propane, butane, butyl acetate, ethyl acetate, ethanol, carbon dioxide, acetone, nitrous oxide, methanol and propan-2-ol, whereby ethanol and carbon dioxide are preferred; ethanol is a particularly preferred food grade solvent. After the extraction step the liquid phase is optionally concentrated or dried by evaporation or freeze drying. The fresh-cut or dried plant or vegetable material may be introduced in cold or preferably hot water and/or solvent, preferably water or a mixture of water with one or more solvents, for a specified period of time, which may vary within wide ranges depending on the kind of plant or vegetable material or solvent used but commonly amounts for example to 1 to 30 minutes, preferably 2 to 15 minutes and most preferred 5 to 10 minutes for a water extraction and for example 30 to 90 minutes, preferably 60 minutes for an ethanol/water extraction. For a water extraction the temperature preferably lies in the range of 85 to 95°C and for an alcohol/water extraction the temperature preferably lies in the range of 55 to 65°C. For a carbon dioxide extraction, the extraction preferably takes place at 0 to 40°C and at super-critical pressure (e.g. 80-200 bar). After the extraction the liquid phase is separated and advantageously concentrated or evaporated to dryness according to known methods. To obtain a concentrated extract two or more extraction steps as described above may be combined. Moreover, the plant or vegetable extracts may be obtained by introducing the fresh-cut or dried plant or vegetable in water and subjecting the mixture to a steam

distillation. The distillate is collected and is then advantageously concentrated or evaporated to dryness.

The extract may be used in liquid form, particularly in aqueous form, or in solid form, particularly in granulate or powder form. If the extract is in liquid form, it has a solid contents of for example from 1 to 25 % by weight, preferably from 2 to 20 % by weight and most preferred from 2 to 15 % by weight.

The amount of inventive plant/vegetable extract or concentrate to be supplied may vary within wide ranges, depending on i.a. the desired treatment, subject to be treated and his needs. Thus, where the subject to be treated is an adult person (typically of ca. 60 to 75 kg body weight), a satisfactory inhibitory effect on bone resorption is, in general obtained with compositions formulated to allow a daily administration of 0.1 to 20 grams, preferably 0.2 to 15 grams and most preferred 0.4 to 10 grams of allium, petroselinum, brassica and/or eruca concentrate or extract (on a solvent-free basis).

Suitable nutritional compositions comprising the above-mentioned plant/vegetable extracts or concentrates represent a further object of the invention. They are characterized in that they comprise

(a) at least one plant/vegetable extract or concentrate selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates,

(b) a calcium source, and

(c) at least one energy source selected from the group consisting of carbohydrate, fat and nitrogen sources, and optionally

(d) Vitamin D.

Regarding component (a), the definitions, preferences and amounts given before for the allium, petroselinum, brassica and eruca extracts and concentrates apply. It is also possible to have a mixture of two or more of said plant/vegetable extracts and concentrates as component (a). The nutritional compositions of the invention conveniently comprise (in % by weight) for example from approximately 0.1 to 40 %, preferably from approximately 3 to 25 % and most preferred from 5 to 15 % of plant/vegetable extract or concentrate component (a).

The calcium source (b) may comprise any physiological acceptable inorganic or organic compound containing calcium. Examples are inorganic calcium salts, for example calcium chloride, calcium phosphate, calcium sulfate, calcium oxide, calcium hydroxide or calcium carbonate, or organic calcium components like whole or skim milk powder, calcium caseinate or calcium salts of organic acids such as calcium citrate, calcium maleate, or mixtures thereof. The use of organic calcium compounds, particularly skim milk powder, calcium caseinate or mixtures thereof, as calcium source (b) is preferred. The amount of calcium component to be supplied may vary within wide ranges. In general, the inventive compositions comprise in one unit dosage from about 100 mg to 1000 mg, preferably 200 mg to 700 mg and most preferred 300 to 600 mg of calcium (on an elemental basis).

The nutritional compositions of the invention conveniently comprise (in % by weight) for example from approximately 1 to 60 %, preferably from approximately 5 to 50 % and most preferred from 10 to 40 % of calcium component (b).

Suitable carbohydrate sources include for example maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and/or sorbit. In these forms the carbohydrates are both energy suppliers and sweeteners. The inventive compositions may contain one or more different carbohydrate sources.

Suitable fat sources include omega-6 polyunsaturated fatty acid sources, omega-3 polyunsaturated fatty acid sources, mono-unsaturated fatty acid sources, medium chain fatty acid sources (i.e. C_6-C_{12}-fatty acids); or mixtures thereof. The above-mentioned fatty acids may be employed in each case in form of the free acid, in mono-, di- or particularly in triglyceride form, or in form of a pharmacological or nutritional acceptable natural source.

Suitable natural sources of omega-6 polyunsaturated fatty acids include vegetable oils such as safflower oil, sunflower oil, soya oil, cotton oil and corn oil. Suitable natural sources of omega-3 polyunsaturated fatty acids include linseed oil and fish oils such as menhaden oil, salmon oil, mackerel oil, tuna oil codliver oil and anchovy oil.

Suitable natural sources of mono-unsaturated fatty acid sources are particularly omega-9 mono-unsaturated fatty acids, for example olives, canola, safflower (hybrids) and sunflower (hybrids).

A preferred fat source comprises triglyceride oils supplying the desired amounts of omega-6 polyunsaturated fatty acids and omega-3 polyunsaturated fatty acids and which are rich in the medium chain fatty acid residues (i.e. residues of C_6-C_{12} fatty acid) and/or mono-unsaturated fatty acid residues. The inventive compositions may contain one or more different fat sources.

Examples of suitable nitrogen sources of the inventive nutritional compositions include sources containing nutritionally acceptable proteins such as soy bean derived proteins; milk proteins such as whey proteins or caseinates; and/or protein hydrolysates; and/or essential amino acids mixtures in free amino acid form or salt form; and/or compounds associated with the synthesis of polyamines, such as arginine, arginine precursors, ornithine and the like, in free amino acid form or salt form.

Preferred nitrogen sources of the nutritional compositions are
(i) soy bean derived proteins, which may be employed in the form of soy beans or in the form of any suitable soja extract or concentrate, for example in form of soy flour, dried soy sprouts, soybean milk, or as dried aqueous extract from soybeans; or
(ii) milk proteins, for example whey derived proteins or caseinates which may be employed for example in the form of whey powder, caseinate salts such as calcium caseinate and/or whole or preferably skim milk powder and/or
(iii) a mixture of essential amino acids and/or
(iv) arginine as nitrogen source.

Milk proteins such as whey powder, caseinates, particularly calcium caseinate, and/or skim milk powder are another particularly preferred nitrogen source of the claimed nutritional compositions. The inventive compositions may contain one or more different nitrogen sources.

The nutritional compositions comprise (in % by weight) for example, from approximately 0.1 % to 98,9 %, preferably from approximately 1 to approximately 95 %, and most preferred from 10 to 90 % of energy source component (c).

The contribution of the nitrogen source, carbohydrate source and fat source to the caloric of the inventive nutritional compositions may vary within wide ranges. For example, the carbohydrate source provides for 30 to 70 % of the total energy supply, the nitrogen source for 5 to 45% and the fat source for 0. 1 to 15 % of the total energy supply of the composition. In preferred compositions of the invention the carbohydrate source provides for 40 to 60 % of the total energy supply, the nitrogen for 20 to 35 % and the fat source for 3 to 12 % of the total energy supply of the composition.

A preferred energy source (c) of the inventive compositions comprises
30 to 70 % of the total energy supply of one or more carbohydrate sources selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and sorbit;
5 to 45 % of the total energy supply of one or more nitrogen sources selected from the group consisting of soy bean derived proteins, milk proteins, a mixture of essential amino acids and arginine and
0. 1 to 15 % of the total energy supply of one or more fat sources comprising omega-3- and omega-6-polyunsaturated fatty acids.

A particularly preferred energy source (c) of the inventive compositions comprises
40 to 60 % of the total energy supply of one or more carbohydrate sources selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and sorbit;
20 to 35 % of the total energy supply of one or more nitrogen sources selected from the group consisting of soy bean derived proteins, skim milk powder and caseinates; and
3 to 12 % of the total energy supply of one or more fat sources comprising omega-3- and omega-6-polyunsaturated fatty acids.

The amount of Vitamin D (optional component (d)) to be supplied may vary within wide ranges. In general, the inventive compositions comprise in one unit dosage from about 400 IU to 1000 IU, preferably about 500 IU.

The nutritional formulations of the invention may comprise other nutritionally acceptable components such as vitamins, minerals, trace elements, fibers (preferably soluble fibers), flavors, preservatives, colorants, sweeteners, emulsifiers and the like.

Examples of vitamins suitable for the incorporation in the composition of the invention include Vitamin A, Vitamin D, Vitamin E, Vitamin K, Vitamin C, folic acid, thiamin, riboflavin, Vitamin B_6, Vitamin B_{12}, niacin, biotin and panthotenic acid in pharmaceutical or nutritionally acceptable form.

Examples of mineral elements and trace elements suitable for the incorporation in the composition of the invention include sodium, potassium, phosphorous, magnesium, copper, zinc, iron, selenium, chromium and molybdenum in pharmaceutical or nutritionally acceptable form.

The term soluble fiber as used herein refers to fibers which are able to substantially undergo fermentation in the colon to produce short chain fatty acids. Examples of suitable soluble fibers include agar-agar, alginates, carubin, carrageenan, gum arabic, guar gum, karaya gum, locust bean gum, pectin, tragacanth, or xanthan gum. They may be hydrolysed or not.

Suitable flavors include natural or artificial flavors, for example fruit flavors such as banana, orange, peach, pineapple or rasberry; vegetable flavors; or vanilla, cocoa, chocolate, coffee and the like.

Preferred ingredients of the inventive nutritious compositions in addition to components (a), (b), (c) and (d) comprise beta-carotene (Vitamin A), Vitamin E, Vitamin C, thiamin, Vitamin B_1, B_6 and/or B_{12}, potassium, magnesium, selenium, zinc, phosphorous and soluble fiber in pharmaceutical or nutritionally acceptable form.

The nutritional compositions may comprise (in % by weight) for example, from approximately 0.1 % to 15 %, preferably from approximately 0.2 to approximately 10 %, and most preferred from 0.5 to 5 % of these additional components other than components (a), (b), (c) and optionally (d).

The inventive nutritional formulations may be formulated and administered in any form suitable for enteral administration, for example oral administration or tube feeding, e.g. nasal administration. The formulations are conveniently administered in the form of an aqueous liquid. The formulations suitable for enteral application are accordingly preferably in aqueous form or in powder or granulate form, whereby the powder or granulate is conveniently added to water prior to use. For use as tube feeding, the amount of water to be added will i.a. depend on the patient's fluid requirements and condition.

The inventive nutritional compositions may be in form of a complete formula diet (in liquid or powder form), such that, when used as sole nutrition source essentially all daily caloric, nitrogen, fatty acids, vitamin, mineral and trace element requirements are met. In general, the daily amount to be supplied to adult persons will lie in the range of 750 to 3500 kcal/day, in particular of 1000 to 2000 kcal/day. However, the inventive nutritional compositions are preferably intended for use as a dietary supplement. The amount of energy supplied by a supplement should not be too excessive, in order not to unnecessarily suppress the patients appetite. The supplement conveniently comprises energy sources in an amount supplying from 50 to 1500 kcal/day, preferably 100 to 900 kcal/day and most preferred 150 to 700 kcal/day.

The nutritional compositions of the invention which are in liquid form, for example in drink form, or preferably in solid form, for example in granulate or powder form, may be obtained in a manner known per se, e.g. by admixing the ingredients and optionally adding water.

The invention further relates to pharmaceutical compositions in single dose unit form comprising

(a) at least one plant/vegetable extract or concentrate selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates, and

(b) a pharmaceutical acceptable carrier.

These pharmaceutical compositions are compositions for enteral administration, such as oral, nasal or rectal administration. Suitable pharmaceutical compositions may be in liquid form or preferably in solid form and comprise (in % by weight) for example, from

approximately 0.001 % to 100 %, preferably from approximately 0.1 to approximately 50 %, active ingredient (a).

The active ingredient (a) is a plant/vegetable extract or concentrate selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates where the above-given definitions and preferences apply. It is also possible to have a mixture of two or more of said plant/vegetable extracts and concentrates a).

Pharmaceutical compositions for enteral administration are, for example, those in single dose unit forms, such as dragées, tablets, capsules or sachets. They are prepared in a manner known *per se*, for example by means of conventional mixing, granulating, confectioning, dissolving or lyophilising processes.

For example, pharmaceutical compositions for oral administration can be obtained by combining the active ingredient with solid carriers, optionally granulating a resulting mixture and processing the mixture or granules, if desired or necessary after the addition of suitable excipients, to form tablets or dragée cores.

Suitable carriers are especially fillers, such as sugars, for example lactose, saccharose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tri-calcium phosphate or calcium hydrogen phosphate, and also binders, such as starch pastes using, for example, corn, wheat, rice or potato starch, gelatin, tragacanth, methylcellulose and/or polyvinylpyrrolidone, and, if desired, disintegrators, such as the above-mentioned starches, and also carboxymethyl starch, cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Excipients are especially flow-conditioners and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol. Dragée cores are provided with suitable, optionally enteric, coatings, there being used *inter alia* concentrated sugar solutions which may contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents or solvent mixtures or, for the preparation of enteric coatings, solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate. Dyes or pigments may be added to the tablets or dragée coatings, for example for identification purposes or to indicate different doses of active ingredient.

Other orally administrable pharmaceutical compositions are hard gelatin capsules and also soft, sealed capsules consisting of gelatin and a plasticiser, such as glycerol or sorbitol. The hard gelatin capsules may comprise the active ingredient in the form of granules, for example in admixture with fillers, such as lactose, binders, such as starches, and/or glidants, such as talc or magnesium stearate, and, if desired, stabilisers. In soft capsules the active ingredient is preferably dissolved or suspended in suitable liquids, such as fatty oils, paraffin oil or liquid polyethylene glycols, it is likewise being possible to add stabilisers.

Suitable rectally administrable pharmaceutical compositions are, for example, suppositories that consist of a combination of the active ingredient with a suppository base material. Suitable suppository base materials are, for example, natural or synthetic triglycerides, paraffin hydrocarbons, polyethylen glycols or higher alkanols. It is also possible to use gelatin rectal capsules which comprise a combination of the active ingredient with a base material. Suitable base materials are, for example, liquid triglycerides, polyethylenglycols or paraffin hydrocarbons.

The inhibitory effect on bone resorption of the inventive plant or vegetable extracts and concentrates may be assessed by measuring the urinary excretion of [^{3}H]-tetracycline from chronically prelabled rats as described in R.C. Mühlbauer and H. Fleisch, Am J Physiol 258, R 679-R689 (1990). The method is based on the characteristics (i) that ^{3}H-labeled tetracycline is deposited in hard tissues during their formation; and (ii) when bone is resorbed, [^{3}H]-tetracycline is released, circulates in blood, and is excreted into the urine where it can be assessed by counting ^{3}H. This is probably due to the fact that [^{3}H]-tetracycline from bone circulates in a form that binds poorly to hydroxyapatite and, therefore, [^{3}H]-tetracycline once liberated from bone, is only poorly reutilized during bone turnover, and because of an efficient renal excretion. The method may be performed as follows: rats are injected subcutaneously twice a week with increasing volumes of a solution containing [^{3}H]-tetracycline starting shortly after birth until the age of about six weeks. At the age of about 50 days, the animals are transferred to individual metabolic cages and every rat is fed with the same amount of a standardized diet for about the three weeks. After that, one group of rats is fed with a purified diet, and another group is fed with the purified diet containing in addition a certain amount of an inventive plant or vegetable concentrate or extract. During the experiments, the animals have free access to demineralized water.

When the rats are about 60 days old, daily 24-hour urine collections are started, and the ^3H contents in urine are determined by liquid scintillation counting. A diagram is then prepared wherein the [^3H]-tetracycline contents in urine of the two groups of rats are plotted as a function of time (days).

Suitable experiments show that the plant or vegetable extracts and concentrates of the invention are capable of considerably decreasing the cumulative [^3H]-tetracycline excretion in urine of intact males and castrated female rats which indicates a high inhibitory effect on bone resorption. Accordingly, the claimed nutritional and pharmaceutical compositions are useful for the treatment and prophylaxis of all kinds of diseases or conditions which are characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis.

The inhibitory effect of the plant or vegetable extracts or concentrates on bone resorption may also be assessed by an *in vitro* assay (described in Example 3) in which ivory slices, onto which freshly isolated osteoclasts have been settled, are incubated with a medium containing the extract or concentrate to be tested. The inhibitory effect on osteoclasts is assessed by counting the osteoclast resorption pits on the ivory slice.

In the following Examples, which illustrate the invention, % are parts by weight unless stated otherwise, and temperatures are given in °C.

Example 1:

The following is an example of a suitable composition of an inventive Supplement in powder form.

Supplement in Powder Form (1 portion)

Content		**65.0**	g
Inventive Extract/Concentrate[1)] including carbohydrates, protein and fiber		**14.5**	g
Protein		20.0	g
including	- Ca-caseinate protein	**8.7**	g
	- skim milk powder	**11.0**	g

Fat		**2.8**	g
including	- omega-6 polyunsaturated acids	1.3	g
	- omega-3 polyunsaturated acids	0.03	g
Carbohydrates	(including inventive extract)	31.0	g
including	- lactose	**16.5**	g
	-maltodextrin	**3.5**	g
Fiber (soluble)		**5.0**	g
Further ingredients		**3.0**	g
including	-Na	230	mg
	-K	500	mg
	-Ca	600	mg
	-Mg	90	mg
	-P	430	mg
	-Cl	350	mg
	-Zn	150	mg
	-Retinol (vitamin A)	0.3	mg
	-Calciferol (vitamin D)	5.0	mcg
	-Tocopherol (vitamin E)	3.0	mg
	-Phylloquinone (vitamin K1)	30.0	mcg
	-Thiamin (vitamin B1)	0.4	mg
	-Riboflavin (vitamin B1)	0.5	mg
	-Pyridoxine (vitamin B6)	0.8	mg
	-Cyanocobalamin (vitamin B12)	0.8	mcg
	-Ascorbic acid (vitamin C)	20.0	mg
	-Biotin	50.0	mcg
	-Folic acid	120.0	mcg
	-Niacinamide	5.0	mg
	-Panthothenic acid	2.0	mg
Energy value		229	kcal

1) a) Extract 1 obtained by extracting 48.3 g dry broccoli for 10 minutes at 89 ± 3°C with 483 ml distilled water and then evaporating the extract to dryness.

b) Extract 2 obtained by extracting 48.3 g dry Italian Parsley for 10 minutes at 89 ± 3°C with 483 ml distilled water and then evaporating the extract to dryness.

c) Extract 3 obtained by extracting 26.4 g dry onions for 10 minutes at 89 ± 3°C with 264 ml distilled water and then evaporating the extract to dryness .

d) Extract 4 obtained by extracting 58.9 g dry onions for 10 minutes at 89 ± 3°C with 589 ml distilled water and then evaporating the extract to dryness, followed by a second extraction of the dried water extract with 324 ml of 85% ethanol/15% water for one hour at 60°C, cooling to room temperature and keeping over night at -20°C, decanting the supernatant, evaporating the alcohol and freeze-drying the extract.

e) Extract 5 obtained by extracting 45.3 g dry onions for one hour at 60°C with 453 ml of 85% ethanol/15% water, filtrating, evaporating the alcohol and freeze-drying the extract.

f) Concentrate 6 obtained by drying fresh bear's garlic and grinding it to a fine powder.

The above supplement may be mixed with water and taken in appropriate concentration between meals.

Example 2: - Effect of H_2O extracts of broccoli, dog-parsley and onion on bone resorption

The effect of the inventive plant or vegetable extracts and concentrates on bone resorption is based on a method as described in R.C. Mühlbauer and H. Fleisch, Am J Physiol 259, R 679-R689 (1990). Bone resorption is monitored by the urinary excretion of ^3H in Wistar rats prelabled from birth for 6 weeks with [^3H]-tetracycline as described in the above-mentioned reference. The rats are then housed in individual metabolic cages and are fed for 10 days with a standard laboratory chow (Kliba 331, Klingentalmühle, Kaiseraugst, Switzerland) containing 1.0 g Ca, 0.7 g P, and 80 IU of vitamin D_3/100 g of food. After this adaptation period, all rats received a diet containing 1.0 g Ca, 1.2 g P, and 80 IU of vitamin D_3/100 g dry weight. This was achieved by adding appropriate amounts of Ca-gluconate and neutral phosphate salts to a basic low calcium, low phosphate diet (Sodi 2134, Klingenthalmühle, Kaiseraugst, Switzerland) in powder form for another 10 days during which urine is collected. Then rats were "pair-fed" receiving 28g of wet food per day. One group (n=6) is switched to a purified diet ("Diet P", Sodi 2160, Klingenthalmühle, Kaiseraugst, Switzerland given as wet food with a water content of 45 ± 2% containing 1.0 g Ca, 1.2 g P, and 80 IU of vitamin D_3/100g dry weight), a second group (n=5) is fed with the purified diet containing

in addition 300 mg extract from broccoli per day (corresponding to 1.0 gram of dry broccoli extracted for 10 minutes at 89 ± 3°C with 10 ml distilled water), a third group (n=5) is fed with the purified diet containing in addition 300 mg extract from Italian Parsley per day (corresponding to 1.0 gram of dry Italian Parsley extracted for 10 minutes at 89 ± 3°C with 10 ml distilled water), and a fourth group (n=5) is fed with the purified diet containing in addition 550 mg extract from onion per day (corresponding to 1.0 gram of dry onion extracted for 10 minutes at 89 ± 3°C with 10 ml distilled water).

After 10 days adaptation without urine collection and a further 10 days with urine collection, the rats are allocated to the different treatment groups. Using the baseline 24 hour [^{3}H]-tetracycline excretion as selection criterion, special care is taken to obtain similar mean initial values for each group. Thereafter, the rats are switched to the purified diet with or without inventive extract and daily 24-hour urine collections are performed over a period of 14 days, and the cumulative [^{3}H]-tetracycline excretion in urine is determined by liquid scintillation counting.

After 14 days of treatment the cumulative bone resorption was 9.2%, 9.5% and 17.5% ($p<0.05$) lower as compared to the control group, in rats daily fed the extracts of broccoli, Italian Parsley and onion respectively.

Example 3: - Effect of a bear's garlic concentrate on bone resorption

The same method as described in Example 2 is used except that treatment lasts only six days and the test group (n=5) is fed with the purified diet containing in addition 1 g of a concentrate from bear's garlic (obtained by drying and grinding fresh bear's garlic). It was found that bear's garlic inhibits bone resorption in male rats by 13.5% ($p<0.05$).

Example 4: - Effect of onion extract on *in vitro* resorption

The effect of an onion extract on *in vitro* resorption is investigated on osteoclast-mediated resorption (as described in Arnett TR, Spowage M, 1996, Modulation of the resorptive activity of rat osteclasts by small changes in extracellular pH near the physiological range. Bone 18:277-279) with the following modifications: instead of using bone wafers, ivory

slices are used as mineral substrate to assess osteoclast resorption pits which are counted under tangential illumination after gold spottering (Vitté C, Fleisch H, Guenther HL. 1996. Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated resorption, Endocrinology 137:2324-2333). In this assay one 4x4 millimeter ivory slice, onto which freshly isolated osteoclasts have been settled is incubated per well of a 48-well plate in 250 µl of a medium containing the extract to be tested during 24 hours at 37°C in a 5% CO_2/air atmosphere. For each dose 8 slices are used. Osteoclasts are harvested from femurs of newborn rats which are, after removing the cartilagenous ends, split in half and chopped transversally. This procedure leads to a cell suspension rich in other cells such as osteoblasts. This permits to test effects in broad conditions, that is, as to both, direct effect on osteoclast and indirect effect on the osteoclast mediated by other cells such as osteoblasts.

The onion extract is obtained by extracting a fine powder of onion for 10 minutes in distilled water (100g/l) at 90°C, filtrating and freeze-drying the filtrate, followed by a second extraction of the dry water extract for one hour at 60°C in 85% ethanol/15% water, cooling to room temperature, keeping over night at -20°C to allow precipitation of unwanted material, decanting the supernatant, evaporating the alcohol and freeze-drying the thus obtained residue, whereby 250 mg freeze-dried onion extract are obtained for each g of dry whole onion.

The onion extract (0.017, 0.17, 1.7 mg onion extract/ml medium) inhibited osteoclast-mediated resorption of dentine in a dose-dependent manner, while the number of tartrate-resistant alcaline phosphatase positive ($TRAP^+$) multinucleated cells (MNC) did not decrease significantly. Thus, the ratio pits/ $TRAP^+$ MNC decreased significantly ($p<0.001$). From this it is concluded that the presence of large numbers of $TRAP^+$ MNC in these cultures despite the additions of onion extract indicate that onion extract is not toxic to these cells but rather inhibits the activity of osteoclasts.

WHAT IS CLAIMED IS :

1. Use of a vegetable extract or concentrate, excluding extracts or concetrates derived from leguminosae and hop, having an inhibitory effect on bone resorption in the preparation of a medicament or nutritional formulation for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis.

2. Use of a plant extract or concentrate selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates or mixtures thereof in the preparation of a medicament or nutritional formulation for the treatment or prophylaxis of a disease or condition which is characterized by increased bone resorption, such as Paget's disease, tumor-induced bone disease or particularly osteoporosis.

3. The use according to claim 1 or claim 2, wherein an allium, petroselinum, brassica and/or eruca extract or concentrate in solid form is employed.

4. The use according to any of claims 1 to 3, wherein the medicament or nutritional formulation is formulated to allow a daily administration of 0.1 to 20 grams of allium, petroselinum, brassica and/or eruca extract or concentrate, each on a solvent-free basis.

5. A nutritional composition comprising
(a) at least one plant extract or concentrate selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates,
(b) a calcium source, and
(c) at least one energy source selected from the group consisting of carbohydrate, fat and nitrogen sources, and optionally
(d) Vitamin D.

6. A nutritional composition according to claim 5, wherein the plant extract or concentrate is selected from the group consisting of extracts and concentrates of the botanical species *Allium cepa*, *Allium ascalonicum*, *Allium ursinum*, *Petroselinum crispum*, *Brassica oleracea* and *Eruca sativa*.

7. A nutritional composition according to claim 5 or 6, wherein the plant extract or concentrate is selected from the group consisting of extracts and concentrates from the botanical species *Allium cepa*, *Petroselinum crispum* (in particular *Petroselinum crispum crispum* and *Petroselinum crispum var. neapolitanum*, and *Brassica oleracea* (in particular *Brassica oleracea var. italica*).

8. A nutritional composition according to claims 5 to 7, wherein the plant extract or concentrate is selected from the group consisting of onion, Italian Parsley and broccoli extracts and concentrates.

9. A nutritional composition according to claims 5 to 8, wherein the calcium source (b) is an organic calcium salt.

10. A nutritional composition according to claims 5 to 9, wherein the carbohydrate source in component (c) is selected from the group consisting of maltodextrins, starch, lactose, glucose, sucrose, fructose, xylit and sorbit.

11. A nutritional composition according to claims 5 to 10, wherein the fat source in component (c) is selected from the group consisting of omega-6 polyunsaturated fatty acid sources, omega-3 polyunsaturated fatty acid sources, mono-unsaturated fatty acid sources, C_6-C_{12}- fatty acid sources, and mixtures thereof.

12. A nutritional composition according to claims 5 to 11, wherein the nitrogen source in component (c) is one or more selected from the group consisting of soy bean derived proteins; milk proteins, protein hydrolysates, a mixture of essential amino acids and arginine.

13. A nutritional composition according to any of claims 5 to 12, wherein the carbohydrate source provides for 30 to 70 % of the total energy supply, the nitrogen source for 5 to 40 % and the fat source for 0.01 to 5 % of the total energy supply of the composition.

14. A nutritional composition according to any of claims 5 to 13, which comprises (in % by weight) from 3 to 25 % of component (a), from 5 to 50 % of component (b) and from 1 to 95 % of component (c).

15. A nutritional composition according to any of claims 5 to 14, which comprises in addition (in % by weight) 0.2 to 10 % of other nutritionally acceptable components selected from the group consisting of vitamins, minerals, trace elements, fibers, flavors, preservatives, colorants, sweeteners and emulsifiers.

16. A nutritional composition according to any of claims 5 to 15, which is in the form of a dietary supplement having from 50 to 1500 kcal/day.

17. A nutritional composition according to any of claims 5 to 16, which is in liquid form.

18. A nutritional composition according to any of claims 5 to 16, which is in solid form, particularly in granulate or powder form.

19. A pharmaceutical composition in single dose unit form, comprising

(a) at least one plant extract or concentrate selected from the group consisting of allium, petroselinum, brassica and eruca extracts and concentrates, and

(b) a pharmaceutical acceptable carrier.

20. A pharmaceutical composition according to claim 19 for enteral administration which is in the form of a dragée, tablet, capsule, sachet or suppository.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/02627

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 095, no. 003, 28 April 1995 & JP 06 340542 A (SUNTORY LTD), 13 December 1994 see abstract	1
X	PATENT ABSTRACTS OF JAPAN vol. 016, no. 552 (C-1006), 20 November 1992 & JP 04 211609 A (TAKEDA CHEM IND LTD), 3 August 1992 see abstract	1

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet) (July 1992)

EXHIBIT E

than that of spirographis porphyrin dimethyl ester, and therefore can be easily distinguished from spirographis porphyrin. The properties of the two isomers of monoformylmonovinyl porphyrins are similar to those reported by Inhoffen *et al.* (1966, 1969).

The biological and biophysical properties of the iron complexes of these formylporphyrins after recombination with human apohemoglobin will be shown elsewhere (Asakura and Sono, 1974).

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The Isolation and Characterization of γ-L-Glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine Sulfoxide from Sandal (*Santalum album* L). An Interesting Occurrence of Sulfoxide Diastereoisomers in Nature†

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ABSTRACT: γ-L-Glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide (**1**) has been isolated from sandal (*Santal album* L.) where it comprises approximately 0.5% of the weight of the dried leaves. The structure was proved by nuclear magnetic resonance, ir, and circular dichroism spectroscopy, by acid and enzymatic hydrolyses and by comparison with a sample of **1** previously isolated from onion (*Allium cepa*). Circular dichroism measurements established that the sulfoxide group in the sandal and onion peptides are of opposite configurations.

A routine amino acid analysis of sandal (*Santalum album* L.) leaves by two-dimensional chromatography revealed two unknown spots. One was identified as the polyamine, *sym*-homospermidine (Kuttan *et al.*, 1971). Material from the other spot, when examined with the amino acid analyzer, revealed a peak in the region of the acidic amino acids, emerging 15 min before *trans*-4-hydroxyproline. This acidity was exploited in isolating this compound by ion-exchange chromatography. Acid and enzymatic hydrolyses combined with proton magnetic resonance (pmr), circular dichroism (CD), and ir spectroscopy established the structure of the unknown as γ-L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide (**1**).

The sulfoxide diastereoisomer of **1** had previously been isolated from onion (*Allium cepa*) by Virtanen and Matik-

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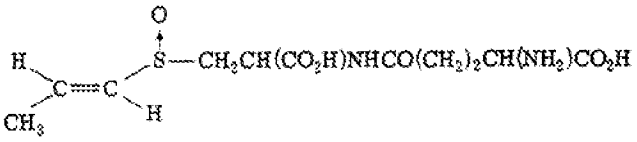
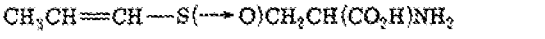


1

kala (1961b,c) who showed that cleavage of the glutamyl peptide bond with a beef kidney preparation yielded the so-called lacrimatory precursor (**2**), also found in onion. When **2** was exposed to an enzyme released from crushed onion, pyruvate, ammonia, and the onion lacrimatory factor (**3**) resulted.



2

3

In the following studies the peptide isolated from sandal is completely characterized and compared with the peptide isolated from onion.

Experimental Section

Materials

Chemicals. The following were obtained as indicated: Dowex 50-X8, H^+, beads, reagent (J. T. Baker); fluoro-2,4-dinitrobenzene (Eastman Kodak Co.); 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma Chemical Co.); *N*-ethylmaleimide (Schwarz Bioresearch); samples of **1** and **2**, isolated from onion, were gifts of Professor A. I. Virtanen (see acknowledgment). All other reagents or solvents used were of analytical or the best available commercial grade.

Instruments. Infrared spectra in KBr discs were obtained with a Perkin-Elmer Model 421 spectrophotometer. Circular dichroism spectra were measured with 0.1–0.2% aqueous solutions of **1** in 0.1-cm cells with a Cary Model 60 spectropolarimeter. Ultraviolet spectra were recorded with a Cary Model 11 spectrophotometer and 1-cm cells. Proton magnetic resonance spectra were obtained in D_2O containing TSP[1] or DSS as internal standards with a Varian Associate HA-100 spectrometer. Chemical shifts are expressed in δ values (ppm) relative to the standard.

Methods

Chromatography. Two-dimensional paper chromatography was performed according to Subramanian and Rao (1955) on Whatman No. 1 paper. Solvent systems used were A, phenol–KCl–HCl buffer (pH 1.0) (50:7 v/v), in the first direction and B, 1-butanol–acetic acid–water (4:1:1) in the second. One-dimensional paper chromatography was done in B, unless otherwise stated. Spots were visualized by spraying with ninhydrin (0.4% in acetone containing 2% 2,4,6-collidine) and by heating at 60° for 10 min. Thin (0.25 mm) layers of silica (F-254 Merck, Darmstadt) and "Avicel" cellulose (Analtech, Wilmington) were developed with B or a 5:2:4 mixture (C), and spots were detected with the uv hand lamp (silica) or a ninhydrin spray (0.1% in methanol–1-butanol–2 N acetic acid (20:10:1)) and brief heating at 100° (silica or cellulose).

Amino acid analyses were carried out according to the method of Benson and Patterson (1965) with the automatic amino acid analyzer (Woods-Jerenberg model) and Beckman custom research resins PA 28 and PA 35.

N-Terminal analysis was done according to the method of Sanger (1945) with fluoro-2,4-dinitrobenzene.

Performic acid oxidation was performed according to the method of Moore (1963).

Hydrolyses were carried out at 100° in sealed tubes with 6 N HCl for 18 hr (complete) or 1 N HCl for 2 hr (partial).

Enzymatic cleavage of **1** was effected with a monkey kidney homogenate prepared following the procedure of Orlowski and Meister (1970) for obtaining γ-glutamyl transferase (EC 2.3.2.1) from beef kidney. Monkey kidney (5 g) was homogenized in the cold with 15 ml of 0.08 M $MgCl_2$ (pH 9.0). An incubation mixture consisting of compound **1** (5 mM) from sandal, $MgCl_2$ (11 mM), Tris-HCl (pH 9.0) (100 mM), and monkey kidney homogenate (0.2 ml) in a total volume of 0.5 ml was kept at 37° for 4 hr. The reaction was then stopped by the addition of 3 ml of ethanol and the mixture centrifuged. The aqueous layer containing the amino acids was separated by the addition of 6 ml of chloroform.

Enzymatic conversion of **2** *to* **3** was achieved by an enzyme preparation from onion (Spare and Virtanen, 1963). Onions (10 g) were homogenized with 30 ml of water in the cold. Endogenous **2** was removed by dialysis against Tris buffer (0.01 M, pH 8.5) and 0.2 ml of this preparation was added to the above hydrolysate.

Isolation. EXTRACTION. Sandal leaves, dried at 50°, were powdered and passed through a 40-mesh sieve; 2.3 kg of powder was extracted by percolation in five batches in a home made extractor with 14 l. of boiling water. The pH of the extract (10 l.) was adjusted to pH 1.5, toluene was added as a preservative, and the extract was allowed to stand overnight at 4°. After removal of a fine precipitate by centrifugation (10^4 g, 10 min), the clear brown supernatant was processed by the ion-exchange procedure below.

FIRST DOWEX 50 COLUMN. The extract was passed through a 4.5 × 130 cm Dowex 50 (H^+ form) column and 500-ml fractions were collected which were monitored for amino acids by the use of paper chromatography and solvent system B. Compound **1**, accompanied by *cis*-4-hydroxyproline, glutamic acid, and aspartic acid, appeared in fraction 17 and thereafter. When the entire extract had passed through the column (fraction 20), the column was washed with 14 l. of water. Fractions 16–21, highly colored, containing **1** and the acidic amino acids were pooled (fraction 1a) while fractions 22–23, light brown, containing mainly **1**, were pooled (fraction 1b), concentrated *in vacuo*, and processed as described below.

SECOND DOWEX 50 COLUMN. Fraction 1b (50 ml) was adjusted to pH 1.0 and passed through a 2.2 × 50 cm Dowex 50 column (H^+ form). After 1 l. of water was passed through the column, 0.25 N ammonium hydroxide was used as eluent and 20-ml fractions were collected. Compound **1** was present, chromatographically pure in fractions 51–63 (pooled fraction 2a). Fractions 64–72 contained **1** contaminated by a few other amino acids (pooled fraction 2b). Fractions 1a and 2b were combined and rechromatographed on a 1-l. Dowex column by the above procedure to obtain another batch of pure **1**.

FURTHER PURIFICATION. The combined batches of **1** were concentrated to 50 ml and 1 l. of acetone was added. The acetone layer was decanted and the precipitated, sticky material was redissolved in 50 ml of water and reprecipitat-

[1] Abbreviations used are: TSP, sodium 3-(trimethylsilyl)propionate-d_4; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate-d_6.

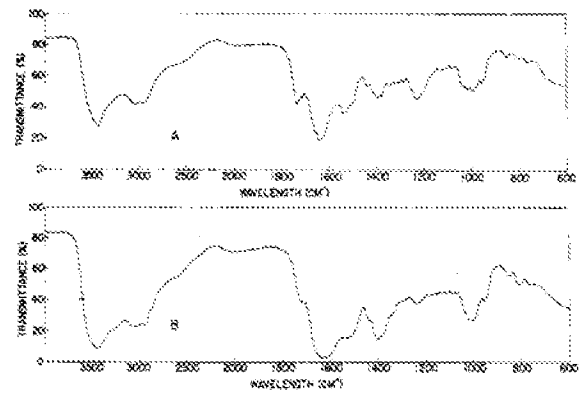


FIGURE 1: Infrared spectra of γ-L-glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide (**1**) from sandal leaves (A) and onion (B). Concentration *ca.* 0.6 mg in 300 mg of KBr.

ed with acetone. This procedure was repeated once more. The precipitate was filtered, washed with 90% acetone, and dried *in vacuo* over P_2O_5 to yield 3.4 g of light brown powder.

CRYSTALLIZATION. To a solution of the above material in 15 ml of water and 100 ml of absolute ethanol was added 200 mg of activated charcoal and the slurry shaken well for 15 min and filtered. The filtrate was heated on a water bath while acetone was added dropwise until a slight turbidity resulted. On cooling to room temperature, then to 4° overnight, a crop of 2.5 g of colorless, granular crystals resulted. This material was recrystallized from aqueous acetone and dried *in vacuo* over P_2O_5 to afford 1.5 g of crystals.

QUANTITATION IN LEAVES. A number of sandal trees near the Wellcome Research Unit were examined for the presence of compound **1**. The leaves from young plants contained only traces while much larger amounts were found in older plants. The amino acid analyzer permitted a quantitative estimation of the peptide in the plant leaves from which the peptide was isolated. Here the peptide constituted nearly 0.5% of the dried leaves. Fresh onions contain nearly 0.2% **1** by weight (Matikkala and Virtanen, 1967), while dehydrated onions contained 0.15% of **2** (Carson *et al.*, 1966).

Results

Physical properties: colorless, granular crystals, mp 140° dec; $[\alpha]^{20}D$ −37.1° (*c*, 0.9%, H_2O). *Anal.* Calcd for $C_{11}H_{18}N_2SO_6 \cdot H_2O$: C, 40.70; H, 6.18; N, 8.65; S, 9.89. Found: C, 41.41; H, 6.05; N, 8.55; S, 9.80.

Spectral Characterization. UV ABSORPTION. Compound **1** did not show any characteristic absorption peak, although a very weak, broad peak centered at 295 nm (ε 67) and a just perceptible shoulder at 230 nm (ε 2.15×10^3) could be observed in water.

IR ABSORPTION. As seen in Figure 1, stretching vibrations for the carbonyl group of a carboxylic acid (1730 cm^{-1}) and an amide (1650, 1530 cm^{-1}), overlapping with a carboxylate carbonyl absorption at 1610 cm^{-1}, are evident. The moderately strong absorptions at 1010 and 955 cm^{-1} may be ascribed to the unsaturated sulfoxide group and a trans-substituted double bond, respectively (Nakanishi, 1962). Absorptions at *ca.* 2500 probably arise from NH_3^+ and the carboxyl O–H stretching frequencies while bands at 3420–2920 (NH_3^+) and 1230 cm^{-1} (C–N stretching?) may arise from an amine function. Also shown on Figure 1 for purposes of comparison is the spectrum of the peptide from onion supplied by Virtanen. Differences in peak intensities may be due to diastereoisomerism or variations in hydration. The onion peptide is extremely hygroscopic. While the elemental analysis of the sandal peptide fits a monohydrate, it did not appear to be particularly hygroscopic.

CD ABSORPTION. Between 280 and 205 nm, a single *negative* maximum was observed for an aqueous solution of the sandal peptide. The molar ellipticity $[\phi]$ at this wavelength (237.5 nm) was -1.36×10^4 ($\Delta\epsilon = -4.12$). The onion peptide in this spectral region exhibited a single *positive* maximum at 237.5 nm, $[\phi] = +1.58 \times 10^4$ ($\Delta\epsilon = +4.79$).

PMR SPECTRA. The sandal peptide in D_2O (DSS) showed the following peaks at 100 MHz: quartet (J = 5.8 Hz) of doublets at 6.71 ($CH_3CH{=}$ (B)), doublet at 6.52 ($=CHS{\rightarrow}O$ (A)), total of two protons, collapsing to an AB quartet centered at 6.61 ($|J_{AB}|$ = 15.2 Hz) on irradiation at center (1.93) of 3-proton allylic methyl doublet, J_{B,CH_3} = 5.8 Hz; a one-proton triplet (J = 6 Hz) centered at 3.90 (Glu α-H), a two proton eight-line multiplet representing the AB (CH_2—S→O) portion of an ABX pattern with δ_B 3.48, δ_A 3.28, J_{AX} = 10, J_{BX} = 4, and J_{AB} = 14 Hz revealed by decoupling the Cys α-H proton (X) by irradiation at the center of a quartet largely hidden by the HOD peak at 4.70; a two-proton multiplet centered at 2.54 (a perturbed triplet, glutamyl CH_2CONH—) and a two-proton broadened triplet at 2.20 (glutamyl β protons; coupled to adjacent glutamyl α-H, $J \simeq 6$ Hz). A nearly identical spectrum resulted for the onion peptide (TSP) with the exceptions that signals for the α protons of Cys and Glu were moved upfield to 4.45 and 3.78, respectively. When the pH of both samples was adjusted to 7.2 (1 N NaOD), this discrepancy disappeared and these signals appeared in both spectra at the positions noted for the onion peptide.

Sodium metabisulfite (100 mg) was added directly to the sandal peptide (44 mg) nmr tube; the tube was shaken well and allowed to stand at 45° for 90 hr. On rescanning, the vinyl signals had shifted upfield and interchanged so that the doublet (J = 15 Hz) appeared at 6.00 while the octet was centered at 5.80. In addition the allylic methyl doublet (J = 5.0) had shifted upfield from 1.93 to 1.70. The bisulfite reduction was 92% complete as judged by the 1.93 doublet (*ca.* 8%) from residual starting material. The eight-line pattern for the cysteinyl methylene signals had also shifted upfield to δ_B 3.24 (quartet) and δ_A 3.04 (quartet) (J_{BX} and J_{AX} remained the same). Signals for the α protons for Cys (4.54 quartet) and Glu (3.92 triplet) as well as the methylene groups of Glu (2.52 and 2.21) were virtually unchanged.

A sample of *S*-(1-propenyl)cysteine sulfoxide (**2**) donated by Professor Virtanen was examined in D_2O (TSP) and the following signals were recorded: octet at 6.78 ($=CHCH_3$, J_{H,CH_3} = 5.5 Hz), doublet at 6.52 ($=CHS{\rightarrow}O$, J = 15 Hz); an AB quartet with δ_B 6.70, δ_A 6.53, J_{AB} = 15.2 Hz resulted from these signals when the center of the allylic methyl doublet (1.95) was irradiated. An eight-line AB portion of an ABX pattern appeared with quartets centered at 3.46 (B) and 3.27 (A) (J_{AX} = 8, J_{BX} = 5.5, and J_{AB} = 14 Hz) and is ascribed to the cysteine methylene group. The α proton (X) gave rise to a quartet at 4.10.

Chemical Properties. Two-dimensional chromatograms of complete hydrolysates of **1** revealed only glutamic acid and cystine. The amino acid analyzer confirmed this and indicated their proportion to be 2:1, respectively. In addition

cystine was identified by its oxidation to cysteic acid as well as reduction to cysteine and reaction with sodium nitroprusside.

Partial hydrolysis gave only glutamic acid. Further hydrolysis (6 N HCl, 18 hr) gave no cystine nor was there any change in the concentration of glutamic acid indicating that partial hydrolysis had completely liberated the glutamic acid and suggesting a γ-glutamyl peptide. The N-terminal was found to be glutamic acid by the fluoro-2,4-dinitrobenzene method.

Performic acid oxidation directly upon **1** produced no cysteic acid; however, cysteic acid did result in oxidation of a complete hydrolysate of the peptide. Compound **1** did not react with sulfhydryl reagents, such as nitroprusside, 5,5'-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide, or ammonium phosphomolybdate, indicating the absence of a free –SH group. Disulfide groups were also absent, since mild reduction produced no detectable –SH groups.

Cystine and glutamic acid isolated from a 6 N HCl hydrolysate were both of the L configuration as indicated by the specific rotation[2] of the former and the observation that the latter reduces NAD^+ in the presence of monkey liver dehydrogenase (Strecker, 1955).

An enzyme preparation from monkey kidney cleaved compound **1** to glutamic acid and the lacrimatory precursor (**2**). The reaction products were identified by paper chromatography in solvent system A in which one component had an R_F (0.16) identical with glutamic acid, while the other had an R_F (0.63) identical with that of **2** obtained from onion. The kidney enzyme hydrolysate on treatment with the onion enzyme yielded pyruvic acid and the lacrimator **3**, identified by reaction with 2,4-dinitrophenylhydrazine (Friedemann, 1957) and a pronounced lacrimatory effect (Spare and Virtanen, 1963), respectively. The lacrimatory effect was checked with a number of volunteers from the Wellcome Research Unit and in each case there was unequivocal evidence for lacrimation compared to control tubes without **1**.

Discussion

The γ-L-glutamyl peptide (**1**) of *S*-(1-propenyl)-L-cysteine sulfoxide (**2**) is the principal γ-glutamyl peptide[3] of onion (*Allium cepa*) (Virtanen and Matikkala, 1961b,c) being accompanied by lesser amounts of γ-glutamyl-*S*-(2-carboxypropyl)cysteine (**4**) (Matikkala and Virtanen, 1970) and -*S*-methylcysteine (Virtanen and Matikkala, 1961a) among others (Virtanen and Matikkala, 1960a,b, 1961a; Matikkala and Virtanen, 1967). γ-Glutamyl peptides of S-alkylated cysteine are also found in garlic (*Allium sativum*) and chives (*Allium shoenoprasum*) and, while related to **1**, exhibit interesting structural differences. Thus chives contain **5**, the reduced, thioether analog of **1** (Virtanen and Matikkala, 1962; Matikkala and Virtanen, 1962), as well as γ-glutamyl-*S*-propylcysteine (Matikkala and Virtanen, 1963), while garlic contains the latter (Virtanen *et al.*, 1962) accompanied by γ-glutamyl-*S*-allylcysteine (Virtanen and Mattila, 1961a).

$RSCH_2CH(CO_2H)NHCO(CH_2)_2CH(NH_2)CO_2H$

4, R = $CH_3CH(CO_2H)CH_2$

5, R = $CH_3CH{=}CH$

Simple cysteine derivatives, *e.g.*, *S*-methyl-, *S*-propyl-, and *S*-(1-propenyl)cysteine, occur in onion in both the thioether and sulfoxide form, the latter in garlic in the sulfoxide form (Sugii *et al.*, 1963), and *S*-allylcysteine as the thioether in onion and the sulfoxide in garlic,[4] *but the γ-glutamyl peptides of these cysteine derivatives are rarely found in the oxidized state* (Virtanen, 1965). The single exception seems to be the occurrence in lima beans of γ-glutamyl-*S*-methylcysteine sulfoxide as a minor component accompanying the unoxidized peptide (Rinderknecht, 1957, 1958). This same sulfoxide which is also found in small amounts with γ-glutamyl-*S*-methylcysteine in red kidney bean extracts may be an artifact arising on air oxidation either during the extraction or during paper chromatography (Zacharius *et al.*, 1959). Virtanen and Matikkala ruled out (1961c) the possibility that **1** was an oxidative artifact of **5** by showing that **2** prepared from **1** by action of a beef kidney enzyme has the same rotation and hence the same sulfoxide configuration as **2** isolated crystalline from onion. Our circular dichroism spectrum of **1** from onion corroborates a highly stereospecific and presumably enzymatic oxidation of some precursor (*e.g.*, **5** or *S*-(1-propenyl)cysteine) in onions. Likewise, we conclude that the sandal peptide **1** originates by a stereospecific oxidation, in this case, to a sulfoxide configuration opposite to that of the onion peptide.

For our structure proof, we relied upon chemical hydrolysis to glutamic acid and cystine. Milder acid hydrolysis liberated glutamic acid and indicated the presence of a γ-glutamyl linkage in the peptide, a peptide in which glutamic acid was also N-terminal as indicated by the DNP assay. At this point, spectroscopic data indicated that the peptide had structure **1**. Enzymatic hydrolysis with a monkey kidney preparation, then exposure of this hydrolysate to an onion enzyme extract, liberated a lacrimator, presumably **3**.[5] Fi-

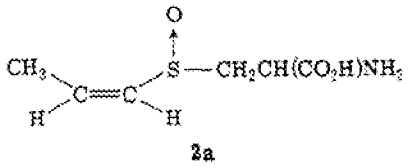
[2] A cystine sample obtained by combined preparative paper (solvent B) and ion-exchange chromatography was contaminated by a ninhydrin-negative, positive-rotating impurity. The observed rotation $[\alpha]^{20}D$ −63° (*c* 1.8, 5 N HCl), however, supports the L configuration (lit. −232° (Meister, 1965)). Pmr (D_2O) and ir (KBr) spectra indicated the sample to be substantially cystine. The presence of appreciable *meso*-cystine is ruled out by the automatic amino acid analyzer.

[3] Two reviews (Fowden, 1964; Waley, 1966) discuss the occurrence of γ-glutamyl peptides in plants.

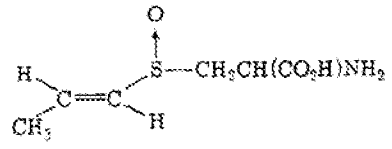
[4] This sulfoxide (alliin) was isolated by Stoll and Seebeck (1948, 1949) and shown to be the precursor of the potent bactericide allicin ($CH_2{=}CHCH_2S(\rightarrow O)SCH_2CH{=}CH_2$) under the action of garlic alliinase. Alliin with a dialyzed onion extract does not generate a lacrimator (Spare and Virtanen, 1963, Virtanen, 1965). Alliinase from either garlic (Stoll and Seebeck, 1951) or onion (Schwimmer and Mazelis, 1963) shows the same preference for (+) over (−) diastereoisomers of L-cysteine sulfoxide as exhibited by the C–S lyases (*Albizzia lophanta*, Schwimmer and Kjaer, 1960; *Allium cepa*, Schwimmer *et al.*, 1964; broccoli, Mazelis, 1963) which have been studied, although the latter have much reduced substrate specificity. In view of the fact that **1** from onion and sandal are of opposite configuration at the sulfoxide group, the derived lacrimatory precursors (**2**) must also be diastereoisomers. At this time we have not yet studied the kinetics for the production of **3** from **2** (sandal) using the onion enzyme.

[5] The propenylsulfinic acid structure $CH_3CH{=}CHSH{\rightarrow}O$ was originally proposed for **3** by Virtanen and Spare (Virtanen and Spare, 1962; Moisio *et al.*, 1962; Spare and Virtanen, 1963). This has been revised to the thiopropanal oxide structure, a sulfine, as a result of work by Wilkens (1961) and Brodnitz and Pascale (1971). Consistent with this structure is the observation of Carson and coworkers that either of the sulfoxide diastereoisomers (**2a**) from synthetic *S*-(*cis*-1-propenyl)-L-cysteine produces a lacrimator when exposed to an onion enzyme preparation (Carson and Wong, 1963; Carson and Boggs, 1966). It should also be mentioned that geometric isomers are possible for **3** (*i.e.*, **3a**, **3b**) (*cf.* King and Durst, 1966; Tangerman and Zwanenburg, 1973). Consequently, unless a rapid room temperature equilibration intervenes, one sulfoxide diastereoisomer of either **2a** or **2b** should generate cis lacrimator **3a** while the other diastereoisomers should produce the trans isomer **3b**.

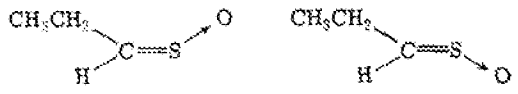
nally the onion and sandal peptides had identical R_F's on thin layers of cellulose (0.16, solvent B; 0.54, solvent C) or silica (0.21, solvent C).



2a



2b

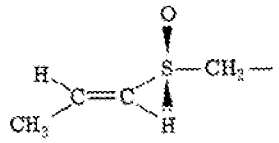


3a **3b**

Summary of the Spectral Data. The proton magnetic resonance spectrum of **1** from sandal provides unambiguous evidence that the alkyl group attached to cysteine is an *S*-(1-propenyl) group with trans configuration. An allylic methyl group (δ 1.93) coupled (J = 5.8 Hz) to an adjacent vinyl proton (δ 6.71) which is in turn coupled to a second vinyl proton (δ 6.52, CHS→O) was observed. The coupling constant (J = 15 Hz) of this latter interaction strongly supports a trans orientation of the two vinyl protons (*cf.* Carson *et al.*, 1966). The sizable upfield shifts for the vinyl quartet ($\Delta\delta$ = 0.91), the vinyl doublet ($\Delta\delta$ = 0.52), the cysteine methylene group ($\Delta\delta$ = *ca.* 0.30), and the allylic methyl ($\Delta\delta$ = 0.23)—all signals from protons in close proximity to the cysteine sulfur atom—on treatment with bisulfite, a reagent known to effect the reduction of sulfoxides (Micheel and Schmitz, 1939), support a sulfoxide group in **1**. The vinyl proton (δ 6.67) adjacent to the sulfoxide group in methyl vinyl sulfoxide shifts upfield by 0.25 ppm on reduction of the sulfoxide group (Chapman and Magnus, 1966). The trans-substituted double bond and the sulfoxide group derive further support from the moderately strong ir absorptions observed at 955 and 1010 cm^{-1}, respectively (*cf.* Spare and Virtanen, 1963; Zacharius *et al.*, 1959). Finally the good correspondence between the negative CD maximum at 237.5 nm, a shoulder observed at approximately 230 nm in the uv, and Karrer and coworkers' report (1951) on the uv absorption of $C_{12}H_{25}S(\rightarrow O)CH{=}CH_2$ ($\lambda_{sh}^{H_2O}$ 230 nm; ϵ 2.8 × 10^3) and the natural product sulforaphen, $CH_3S(\rightarrow O)CH{=}CH{-}(CH_2)_3N{=}C{=}S$ (λ_{max}^{EtOH} 231 nm; ϵ 3.24 × 10^3) provide additional evidence for a sulfoxide grouping with an adjacent unsaturated group. A saturated sulfoxide would absorb at somewhat shorter wavelengths (*ca.* 190–210 nm; Mislow *et al.*, 1965).

Virtanen and Spare, in establishing the structure of **2** (Virtanen and Spare, 1962; Moisio *et al.*, 1962; Spare and Virtanen, 1963), left unresolved the configuration of the propenyl group. Recently Carson and coworkers have shown (Carson *et al.*, 1966) by pmr that the configuration of **2** which they isolate from onion is clearly trans (*i.e.*, **2b**). We have examined by pmr samples of **1** and **2** isolated by the Helsinki group from onion as well as **1** isolated from sandal and have found (see Results section) that all possess a trans propenyl group. In addition samples of **1** from either onion or sandal had virtually identical pmr spectra; in this instance diastereoisomers cannot be clearly differentiated by pmr.

The oxidation of the presumed precursor **5** to give **1** in onion or sandal is apparently stereospecific as judged by the positive and negative maxima observed in their respective circular dichroism spectra. One of the two possible sulfoxide configurations must be the sole or predominant product in either case since the absolute values of the molar ellipticities are approximately the same. Such stereospecificity is apparently not usual in the onion, however, for racemic sulfoxide mixtures occur in the case of *S*-methyl- and *S*-propyl-L-cysteine and L-methionine (Matikkala and Virtanen, 1967). If the same assignments (Mislow *et al.*, 1965) obtain for both *S*-propenyl and for methyl alkyl sulfoxides, a positive CD maximum at 237.5 nm would indicate an *S* configuration for the onion sulfoxide group, while a negative maximum at that wavelength would correspond to an *R* configuration for the sandal sulfoxide group. The *R* configuration is shown below (**1a**) for the sandal peptide. Mislow



1a

and coworkers (Axelrod *et al.*, 1968a,b) have cautioned on extending a rule worked out for simpler sulfoxides to systems where a sulfoxide substituent could perturb the sulfoxide $n\rightarrow d$ transition.[6] Such would certainly be the case for the unsaturated *S*-propenyl group; consequently, the above assignments must be regarded as provisional. Hermann and coworkers (1971) have shown that the positive and negative CD maxima at approximately 220 nm which result from the two N^{α}-acetyl-L-thialysine sulfoxide diastereoisomers correspond to the *S* and *R* configurations, respectively, of the sulfoxide group and that amino acid and amide carbonyl absorptions can be ignored. A similar conclusion had been reached earlier by Gaffield and coworkers (1965) with regard to the L-amino acid contributions to the positive and negative ORD curves of *S*-alkyl-L-cysteine sulfoxide diastereoisomers.

Interestingly most of the optically active sulfoxides which have been isolated from natural sources and whose sulfoxide configurations have been determined are of the *S* configuration (Lucas and Levenbook, 1966; Barnsley, 1968). The single exception seems to be the class of isothiocyanate sulfoxides, $CH_3S(\rightarrow O)(CH_2)_nNCS$ (n = 3–6; 8–10) and sulforaphene (see above), found in mustard oil (Cheung *et al.*, 1965).

The various γ-glutamyl peptides including **1** disappear from the bulbs of sprouting onion and garlic and these may therefore function as nitrogen reserves (Virtanen and Matikkala, 1960b; Virtanen, 1962, 1965). No other role has apparently been proposed for these unusual peptides (see also Fowden, 1964). The occurrence of **1** in a higher plants unrelated to the *Allium* genus is surprising as is the finding that the peptide concentration is *greater* in mature plants than in young plants. The function of **1** in sandal is thus even more obscure.

Bulbs and seeds of onion and garlic lack either a γ-glutamyl peptidase or transferase capable of cleaving the γ-glu-

[6] Allyl-substituted sulfoxides were observed to depart from the rule in isooctane; in water, however, their behavior was normal.

tamyl bond in peptides of either *S*-propenyl- or *S*-allylcysteine sulfoxide, although a γ-glutamyl peptidase is present in *sprouting* bulbs and in germinating seeds of chives (Matikkala and Virtanen, 1965). Subsequent action of alliinase on the cleaved peptide results in C–S bond cleavage and deamination, the products being pyruvate, ammonia, and either the lacrimatory factor (**3**) (onion) or allicin (garlic). In the case of sandal, no lacrimation was noticeable when handling leaves, homogenates, or aqueous extracts so it is possible that the enzymes for converting **1** → **2** → **3** are absent in the leaves.

Peptide **1** in sandal probably originates from the reaction of glutathione with methacrylic acid or some equivalent (Suzuki *et al.*, 1962) followed by cleavage of glycine to **4**, an oxidative decarboxylation to **5**, and, lastly, oxidation to the sulfoxide. Alternatively, cysteine might trap methacrylic acid, receiving at some point in the pathway a glutamyl group from glutathione in a nonspecific transpeptidation (*cf.* Fowden, 1964). These pathways were postulated (Virtanen, 1962, 1965) for **1** and related compounds in onion and received support from the isolation of *S*-(2-carboxypropyl)cysteine -γ-glutamylcysteine (**4**) and -glutathione from onion (Matikkala and Virtanen, 1967, 1970; Virtanen and Matikkala, 1960a,b, 1961a,b).

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A Potent Interferon Inducer Derived from Poly(7-deazainosinic acid)†

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ABSTRACT: To determine whether or not purine N-7 of poly(I) plays a significant role in the induction of interferon by poly(I) · poly(C), poly(7-deazainosinic acid)[poly(c^7I)] was prepared by the *Micrococcus luteus* polynucleotide phosphorylase catalyzed polymerization of 7-deazainosine 5′-diphosphate, synthesized from 7-deazainosine (7-(β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ol). Poly(c^7I) was, like poly(I), degraded to the nucleoside or nucleotide level by T_1 ribonuclease, bovine spleen phosphodiesterase, snake venom phosphodiesterase, micrococcal nuclease, and 0.3 N KOH but was totally resistant to degradation by pancreatic ribonuclease A. Unlike poly(I), poly(c^7I) showed little temperature-dependent hyperchromicity in 1.0 M NaCl with an indication of structure only below room temperature. Mixing curves as a function of wavelength, isosbestic points, and sedimentation velocity studies demonstrated that poly(c^7I) forms only 1:1 stoichiometric complexes with both poly(C) and poly(br^5C). Poly(C) · poly(c^7I) had a T_m of 49° (0.2 M NaCl, pH 7) and poly(br^5C) · poly(c^7I) had a T_m of 86° (0.2 M NaCl, pH 7). For comparison purposes, the previously reported poly(br^5C) · poly(I) complex was also prepared. These complexes were evaluated for antiviral activity and interferon inducing ability. With primary rabbit kidney cells, the following sequence (in order of decreasing activity) was established when direct inhibition of vesicular stomatitis virus cytopathogenic effect and interferon production in normal, interferon primed and superinduced (cycloheximide and actinomycin D) rabbit kidney cells were measured: poly(c^7I) · poly(br^5C) > poly(I) · poly(br^5C) > poly(I) · poly(C) > poly(c^7I) · poly(C). On the other hand, if the components of the complexes were administered sequentially followed by measurement of inhibition of virus cytopathogenic effect, the sequence (in order of decreasing activity) changed to: poly(I) · poly(C) > poly(c^7I) · poly(br^5C) > poly(I) · poly(br^5C) > poly(c^7I) · poly(C), if either poly(I) or poly(c^7I) were added to the cells first. If either poly(br^5C) or poly(C) were administered first, the order of decreasing activity was: poly(I) · poly(C) > poly(I) · poly(br^5C) > poly(c^7I) · poly(br^5C) > poly(c^7I) · poly(C).

The synthesis and biological evaluation of a number of modified polynucleotides have rendered possible the definition of several structural features required for an effective interferon inducer (Vilcek *et al.*, 1968; Colby and Chamberlin, 1969; De Clercq *et al.*, 1969, 1970, 1972a, 1974b; Steward *et al.*, 1972; Black *et al.*, 1972; Torrence *et al.*, 1973a,b; De Clercq and Janik, 1973). While a number of modifications have involved the pyrimidine base of poly(I) · poly(C) (Colby and Chamberlin, 1969; De Clercq *et al.*, 1972a; Reuss, K. and Scheit, K. H., personal communication, 1973; Folayan and Hutchinson, 1974; Johnston *et al.*, 1974) or poly(A) · poly(U) (Torrence *et al.*, 1973a; De Clercq *et al.*, 1974b), with one exception (De Clercq *et al.*, 1974), no nuclear modification involving the purine base of either complex has been reported. Since there is evidence to indicate that the purine member of poly(I) · poly(C) may be of greater importance in the induction process (De Clercq and De Somer, 1972; Carter *et al.*, 1972; Mohr *et al.*, 1972; De Clercq *et al.*, 1973), we have initiated an investigation into the effects of such nuclear modifications on the ability of polynucleotides to function as interferon inducers. In this paper, we report the synthesis, physical properties, and biological activity of one such modified polynucleotide in which N-7 of the hypoxanthine base of poly(I)[1]

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[1] Abbreviations for synthetic polynucleotides conform to the recommendations of the IUPAC-IUB Commission ((1970), *Biochemistry 9,* 4025). Thus poly(I) represents poly(inosinic acid), poly(I) · poly(C) represents the two-stranded complex with poly(C), poly(c^7I) is poly(7-deazainosinic acid), poly(c^7A) is poly(7-deazaadenylic acid), etc. Other abbreviations are as follows: T_m, temperature at the midpoint of the absorbancy change; CPE, cytopathogenic effect; PRK cells, primary rabbit kidney cells; VSV, vesicular stomatitis virus; MEM, minimal Eagle's medium; MIC, minimum inhibitory concentration; PFU, plaque forming units.

EXHIBIT F

Display Settings: Abstract

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A gamma-glutamyl peptide isolated from onion (Allium cepa L.) by bioassay-guided fractionation inhibits resorption activity of osteoclasts.

Wetli HA, Brenneisen R, Tschudi I, Langos M, Bigler P, Sprang T, Schürch S, Mühlbauer RC.

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Abstract

One gram of onion added to the food of rats inhibits significantly ($p < 0.05$) bone resorption as assessed by the urinary excretion of tritium released from bone of 9-week-old rats prelabeled with tritiated tetracycline from weeks 1 to 6. To isolate and identify the bone resorption inhibiting compound from onion, onion powder was extracted and the extract fractionated by column chromatography and medium-pressure liquid chromatography. A single active peak was finally obtained by semipreparative high-performance liquid chromatography. The biological activity of the various fractions was tested in vitro on the activity of osteoclasts to form resorption pits on a mineralized substrate. Medium, containing the various fractions or the pure compound, was added to osteoclasts of new-born rats settled on ivory slices. After 24 h of incubation, the tartrate-resistant acid phosphatase positive multinucleated cells, that is, osteoclasts, were counted. Subsequently, the number of resorption pits was determined. Activity was calculated as the ratio of resorption pits/osteoclasts and was compared to a negative control, that is, medium containing 10% fetal bovine serum only and to calcitonin (10(-12) M) as a positive control. Finally, a single peak inhibited osteoclast activity significantly ($p < 0.05$). The structure of this compound was elucidated with high-performance liquid chromatography-electrospray ionization-mass spectrometry, time-of-flight electrospray ionization mass spectrometry, and nuclear magnetic resonance spectroscopy. The single peak was identified as gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide (GPCS). It has a molecular mass of 306 Da and inhibits dose-dependently the resorption activity of osteoclasts, the minimal effective dose being approximately 2 mM. As no other peak displayed inhibitory activity, it likely is responsible for the effect of onion on bone resorption.

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